PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
80021-211		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/CA 00/ 00907	04/08/2000	04/08/1999

Applicant

THE UNIVERSITY OF BRITISH COLUMBIA

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[] contained in the international application in written form.

[] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [] **Certain claims were found unsearchable** (See Box I).

3. [] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

[X] as suggested by the applicant.

[] because the applicant failed to suggest a figure.

[] because this figure better characterizes the invention.

[] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/00907

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, STRAND, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 15387 A (LARDIZABAL KATHRYN DENNIS ;LASSNER MICHAEL W (US); METZ JAMES GEOR) 8 June 1995 (1995-06-08) the whole document ---	1,4,6,7, 10
X	WO 96 13582 A (DNA PLANT TECHN CORP) 9 May 1996 (1996-05-09) cited in the application examples 2,3 ---	1,4,6,7, 10
Y	ROSCOE TJ ET AL: "Brassica napus fatty acid elongase (FAE1) homolog mRNA" EMBL NUCLEOTIDE SEQU, XP002109729 accession no. U50771 --- -/--	1,4,6,7, 10,13-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 March 2001	02/04/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Holtorf, S

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
Y	VENKATESWARI J ET AL: "Molecular cloning and characterization of FATTY ACID ELONGATION1 (BjFAE1) gene of Brassica juncea." JOURNAL OF PLANT BIOCHEMISTRY AND BIOTECHNOLOGY, vol. 8, no. 1, January 1999 (1999-01), pages 53-55, XP000991381 ISSN: 0971-7811 figure 3 ---	1,4,6,7, 10,13-15
A	WO 98 54954 A (POST BEITTENMILLER MARTHA ANN ;CARGILL INC (US); JAWORSKI JAN G (U) 10 December 1998 (1998-12-10) example 1 ---	
A	JAMES D W ET AL: "DIRECTED TAGGING OF THE ARABIDOPSIS FATTY ACID ELONGATIONI (FAE1) GENE WITH THE MAIZE TRANSPOREN ACTIVATOR" PLANT CELL,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 7, March 1995 (1995-03), pages 309-319, XP002911493 ISSN: 1040-4651 cited in the application abstract; figure 6 ---	
A	DATABASE EMBL SEQUENCE DATABASE 'Online! 4 July 1995 (1995-07-04) JAMES, D.W.; ET AL. : "DIRECTED TAGGING OF THE ARABIDOPSIS FATTY ACID ELONGATIONI (FAE1) GENE WITH THE MAIZE TRANSPOson ACTIVATOR" XP002163429 accession no. U29142 ---	
A	KUNST L ET AL: "FATTY ACID ELONGATION IN DEVELOPING SEEDS OF ARABIDOPSIS THALIANA" PLANT PHYSIOLOGY AND BIOCHEMISTRY,FR,GAUTHIER-VILLARS, PARIS, vol. 30, no. 4, 1992, pages 425-434, XP002063741 ISSN: 0981-9428 cited in the application ---	
A	WO 98 46766 A (UNIV BRITISH COLUMBIA) 22 October 1998 (1998-10-22) page 5, line 25-27; page 9, line 35; page 10, line 34 - page 11, line 33; example 3; --- -/--	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	MILLAR ANTHONY A ET AL: "CUT1, an Arabidopsis gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme." PLANT CELL, vol. 11, no. 5, May 1999 (1999-05), pages 825-838, XP002163221 ISSN: 1040-4651 page 827, left column; last sentence; right column, first paragraph; Fig.3; ---	
A	WO 99 03983 A (CARNEGIE INST OF WASHINGTON ;MONSANTO COMPANY INC (US)) 28 January 1999 (1999-01-28) the whole document ---	
A	DATABASE EMBL SEQUENCE LIBRARY 'Online! 24 July 1997 (1997-07-24) CLEMENS, S. AND KUNST, L.: "isolation f a Brassica napus cDNA (accession no. AF009563) encoding 3-ketoacyl-CoA synthase, a condensing enzyme involved in the biosynthesis of very long chain fatty acids in seeds (PGR97-125)" XP002163222 accession no. AF009563 ---	
P,X	WO 99 54471 A (HOLROYD GEOFFREY HEYS ;ZENECA LTD (GB); GRAY JULIE ELIZABETH (GB);) 28 October 1999 (1999-10-28) the whole document -----	1,2,4, 6-8,10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 00/00907

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9515387 A	08-06-1995	US 5679881 A	21-10-1997
		CA 2177598 A	08-06-1995
		EP 0731840 A	18-09-1996
		JP 9505739 T	10-06-1997
WO 9613582 A	09-05-1996	AU 703957 B	01-04-1999
		AU 3969995 A	23-05-1996
		CA 2203754 A	09-05-1996
		EP 0788542 A	13-08-1997
		US 6124524 A	26-09-2000
WO 9854954 A	10-12-1998	AU 7721098 A	21-12-1998
		EP 0986296 A	22-03-2000
WO 9846766 A	22-10-1998	AU 7019198 A	11-11-1998
		EP 0975767 A	02-02-2000
WO 9903983 A	28-01-1999	US 5965793 A	12-10-1999
		AU 8404498 A	10-02-1999
		BR 9811014 A	26-09-2000
		EP 1002067 A	24-05-2000
WO 9954471 A	28-10-1999	AU 3615199 A	08-11-1999
		BR 9909765 A	19-12-2000
		EP 1080196 A	07-03-2001

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(19) World Intellectual Property Organization
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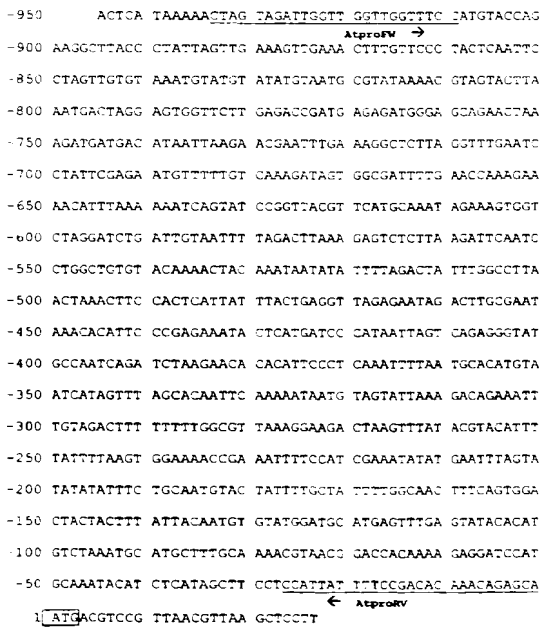
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[Continued on next page]

(54) Title: REGULATION OF EMBRYONIC TRANSCRIPTION IN PLANTS



(57) **Abstract:** Nucleic acid constructs are provided comprising transcriptional regulatory regions homologous to plant *FAE1* promoters. In some embodiments, these constructs may be used in transgenic cells or plants to promote expression of foreign and endogenous genes in developing seeds, for example to affect seed lipid metabolism, protein or carbohydrate composition and accumulation, or seed development.

WO 01/11061 A2

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

REGULATION OF EMBRYONIC TRANSCRIPTION IN PLANTS

FIELD OF THE INVENTION

The invention is in the field of nucleic acid sequences capable of regulating transcription, particularly sequences that may promote transcription during embryogenesis in plants.

BACKGROUND OF THE INVENTION

Most of the information about seed-specific gene expression comes from studies of genes encoding seed storage proteins like napin, a major protein in the seeds of *Brassica napus*, or conglycinin of soybean. Upstream DNA sequences directing strong embryo-specific expression of these storage proteins have been used successfully in transgenic plants to manipulate seed lipid composition and accumulation (Voelker et al., 1996). However, expression of storage protein genes begins fairly late in embryogenesis. Thus, promoters of seed storage protein genes may not be ideal for all seed-specific applications. For example, storage oil accumulation commences significantly before the highest level of expression of either napin (Stalberg et al., 1996) or conglycinin (Chen et al., 1988) is achieved. It is, therefore of interest to identify other promoters which may modulate expression of genes in developing plant embryos.

A variety of transcriptional regulatory regions that may be active during plant embryogenesis are known, as disclosed for example in: U.S. Patent No. 5,792,922 issued 11 August 1998 to Moloney; U.S. Patent No. 5,623,067 issued 22 April 1997 to Vandekerckhove et al.; International Patent Publication WO9845461 published 15 October 1998. There remains a need for alternative transcriptional regulatory regions.

FATTY ACID ELONGATION1 (*FAE1*) genes encode condensing enzymes involved in plant very long chain fatty acid biosynthesis. The FAE1 condensing enzyme is thought to be localized in the endoplasmic reticulum where it catalyzes the sequential elongation of C18 fatty acyl chains to C22 in length (Kunst et al., 1992). *FAE1* genes have been cloned and described recently by James et al. (1995), International Patent Publication WO 96/13582.

SUMMARY OF THE INVENTION

In one aspect, the invention provides transcriptional regulatory regions derived from *FAE1* genes. The transcriptional regulatory regions of the invention may be useful in

promoting early seed-specific transcription of heterologous sequences to which they are operably linked. The transcriptional regulatory regions of the invention may be used in a wide variety of plants, including *Brassica sp.*, *Arabidopsis* and other plant species. DNA constructs comprising the transcriptional regulatory sequences of the invention may be active during fatty acid or lipid biosynthesis in the plant embryo. Certain embodiments of the constructs of the invention may be used in transgenic plants to promote expression of heterologous sequences in developing seeds. In various embodiments, the constructs of the invention may be used to mediate gene expression that affects seed lipid metabolism, or seed protein composition or seed carbohydrate composition, or seed development. In alternative embodiments, the transcriptional regulatory regions of the invention may also be useful for the production of modified seeds containing novel recombinant proteins which have pharmaceutical, industrial or nutritional value.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a 934 bp DNA sequence comprising the *Arabidopsis thaliana FAE1* transcription regulatory sequence.

Figure 2 shows a 1588 bp DNA sequence comprising the *Brassica napus FAE1* transcription regulatory sequence.

Figure 3 shows a 1069 bp DNA sequence comprising the *Lunaria annua FAE1* transcription regulatory sequence.

Figure 4 shows an alignment of the *Arabidopsis thaliana* (*A.t.*), *Lunaria annua* (*L.a.*) and *Brassica napus* (*B.n.*) transcription regulatory sequences. Asterisks below the sequences indicate identical nucleotides in each of the three sequences. A number of putative cis-acting sequence motifs are identified in the *A. thaliana* sequence: an EM1 ABA box at -44bp to -36bp having the sequence ACATCTCAT, for which the published consensus sequence is ACGTGTCAT (Rowley, D.L. and Herman, E.M. (1997), Biochimica et Biophysica Acta 1345:1-4); an A-300 box at -51bp to -46bp having the sequence TGCAAT, for which the published consensus sequence is TG(T/A/C)AAA(G/T) (Morton et al. (1994) in Seed Development and Germination (Kigel, J. and Gallili, G., eds.) pp. 103-138, Marcel Dekker, New York); G-box 1 at -105 to -100 bp having the sequence CACATG, for which is the consensus sequence is CACCTG, and G-box 2 at -164 to -159 bp having the sequence CAACTT, for which the consensus sequence is CAACTG (Kawogoe, Y. and Murai, N. (1992) Plant J. 2:927-936; CE1 element at -226 to -218 bp having the sequence

TTCCATCGA, for which the consensus sequence is TGCCACCGG, and a CE3 element at -381bp to -369 bp having the sequence ACACATTCCCTC, for which the consensus sequence is ACGCGTGTCCTC (Shen et al., (1996) Plant Cell 8:1107-1119). Not highlighted is a putative RY repeat motif at -53bp to -47bp having the sequence CATGCAA, for which the consensus sequence is CATGCAT (Dickinson et al. (1988) Nucleic Acid Res. 16:371; Lelievre et al. (1992) Plant Physiol. 98:387-391). Also shown, as Con. 4, is a consensus sequence, wherein R=G or A, Y=T/U or C, M=A or C, K=G or T/U, S=G or C, W=A or T/U, B=G or C or T/U, D=A or G or T/U, H=A or C or T/U, V=A or G or C and N=A or G or C or T/U.

Figure 5 shows an alignment of the *Arabidopsis thaliana* (*A.t.*) and *Lunaria annua* (*L.a.*) transcription regulatory sequences. Asterisks below the sequences indicate identical nucleotides in each of the two sequences. The base at position -400 in the *A.t.* sequence is highlighted. The alignment of sequences in both Figure 4 and Figure 5 was accomplished using the CLUSTALW program (version 1.74) for multiple sequence alignments, using a gap open penalty of 15, a gap extension penalty of 6.66 and an IUB DNA weight matrix. Also shown, as Con. 5, is a consensus sequence, wherein R=G or A, Y=T/U or C, M=A or C, K=G or T/U, S=G or C, W=A or T/U, B=G or C or T/U, D=A or G or T/U, H=A or C or T/U, V=A or G or C and N=A or G or C or T/U.

Figure 6 includes two bar graphs illustrating hydroxy fatty acid content of A) *FAE1-FAH12* and B) *napin-FAH12* transgenic seeds, expressed as percentage of total seed fatty acids.

Figure 7 shows an alignment of the *Brassica napus* (*B.n.*) and *Lunaria annua* (*L.a.*) *FEA1* transcription regulatory sequences. Asterisks below the sequences indicate identical nucleotides in each of the two sequences.

Figure 8 shows an alignment of the *Brassica napus* (*B.n.*) and *Arabidopsis thaliana* (*A.t.*) *FEA1* transcription regulatory sequences. Asterisks below the sequences indicate identical nucleotides in each of the two sequences.

DETAILED DESCRIPTION OF THE INVENTION

The recombinant nucleic acid molecules of the invention may comprise a heterologous promoter sequence operably linked to a nucleic acid sequence, wherein the promoter sequence comprises a transcriptional regulatory region capable of mediating seed-specific expression in *Arabidopsis*. The transcriptional regulatory region may be obtainable from a

plant *FAE1* gene. Alterntively, The transcriptional regulatory region may hybridize under stringent conditions to a 5' region of the plant *FAE1* gene. In further alternative embodiments, The transcriptional regulatory region may be at least 70% identical when optimally aligned to the 5' region of the plant *FAE1* gene.

In alternative embodiments, the invention provides isolated nucleic acids comprising the transcriptional regulatory regions of the invention. By isolated, it is meant that the isolated substance has been substantially separated or purified away from other biological components with which it would otherwise be associated, for example *in vivo*. The term 'isolated' therefore includes substances purified by standard purification methods, as well as substances prepared by recombinant expression in a host, as well as chemically synthesized substances.

In the context of the present invention, "transcriptional regulatory region" means a nucleotide sequence capable of mediating or modulating transcription of a nucleotide sequence of interest, when the transcriptional regulatory region is operably linked to the sequence of interest. Conversely, a transcriptional regulatory region and a sequence of interest are "operably linked" when the sequences are functionally connected so as to permit transcription of the sequence of interest to be mediated or modulated by the transcriptional regulatory region. In some embodiments, to be operably linked, a transcriptional regulatory region may be located on the same strand as the sequence of interest. The transcriptional regulatory region may in some embodiments be located 5' of the sequence of interest. In such embodiments, the transcriptional regulatory region may be directly 5' of the sequence of interest or there may be intervening sequences between these regions. The operable linkage of the transcriptional regulatory region and the sequence of interest may require appropriate molecules (such as transcriptional activator proteins) to be bound to the transcriptional regulatory region, the invention therefore encompasses embodiments in which such molecules are provided, either *in vitro* or *in vivo*.

The term "recombinant" means that something has been recombined, so that when made in reference to a nucleic acid molecule the term refers to a molecule that is comprised of nucleic acid sequences that are joined together by means of molecular biological techniques. The term "recombinant" when made in reference to a protein or a polypeptide refers to a protein molecule which is expressed using a recombinant nucleic acid molecule. The term "heterologous" when made in reference to a nucleic acid sequence refers to a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence

to which it is not ligated in nature, or to which it is ligated at a different location in nature. The term "heterologous" therefore indicates that the nucleic acid molecule has been manipulated using genetic engineering, i.e. by human intervention.

Sequences may be derived or obtainable from plant *FAE1* genes by deduction and synthesis based upon the wild-type *FAE1* gene sequences. Derived sequences may be identified in different organisms, for example by isolation using as probes the nucleic acid sequences of the invention. Alternative transcriptional regulatory regions may be derived through mutagenesis or substitution of wild-type sequences, such as the sequence disclosed herein. Derived nucleic acids of the invention may be obtained by chemical synthesis, isolation, or cloning from genomic DNAs using techniques known in the art, such as the Polymerase Chain Reaction (PCR). Consensus sequences, such as those illustrated in Figures 4 and 5 are alternative embodiments of the nucleic acids of the invention, derived from the disclose wild-type *FAE1* gene sequences. Nucleic acids of the present invention may be used to design alternative primers (probes) suitable for use as PCR primers to amplify particular regions of an *FAE1* gene. Such PCR primers may for example comprise a sequence of 15-20 consecutive nucleotides of the sequences of the invention. To enhance amplification specificity, primers of 20-30 nucleotides in length may also be used. Methods and conditions for PCR amplification are described in Innis et al. (1990); Sambrook et al. (1989); and Ausubel et al. (1995). As used herein, the term "probe" when made in reference to an oligonucleotide refers to an oligonucleotide which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are, for example, useful in the detection, identification, amplification and isolation of particular gene sequences. Oligonucleotide probes may be labelled with a "reporter molecule," so that the probe is detectable using a detection system, such as enzymatic, fluorescent, radioactive or luminescent detection systems.

Derived nucleic acids of the invention may also be identified by hybridization, such as Southern or Northern analysis. Southern analysis is a method by which the presence of DNA sequences in a target nucleic acid mixture are identified by hybridization to a labeled probe, comprising an oligonucleotide or DNA fragment of a nucleic acid of the invention. Probes for Southern analysis may for example be at least 15 nucleotides in length. Southern analysis typically involves electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after electrophoretic separation, and transfer of the DNA to nitrocellulose, nylon, or another suitable membrane support for analysis with a radiolabeled, biotinylated, or enzyme-

labeled probe as described in Sambrook *et al.* (1989). Similarly, Northern analysis may be used to identify RNAs that hybridize to a known probe such as an oligonucleotide, DNA fragment, cDNA or fragment thereof, or RNA fragment of a nucleic acid of the invention or a known *FAE1* sequence. The probe may be labeled with a radioisotope such as ^{32}P, by biotinylation or with an enzyme. The RNA to be analyzed may be electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe, using standard techniques well known in the art such as described in Sambrook *et al.* (1989).

In alternative embodiments, a transcriptional regulatory region of the invention may be at least 70% identical when optimally aligned to the 5' region of a plant *FAE1* gene, such as the *Arabidopsis FAE1* gene. In alternative embodiments, the degree of identity may be between 50% and 100%, such as 60%, 80%, 90%, 95% or 99%. When a position in the compared sequence is occupied by the same nucleotide or amino acid, following optimal alignment of the sequences, the molecules are considered to have identity at that position. The degree of identity between sequences is a function of the number of matching positions shared by the sequences. In terms of percentage, identity is the sum of identical positions, divided by the total length over which the sequences are aligned, multiplied by 100.

Various aspects of the present invention encompass nucleic acid or amino acid sequences that are homologous to other sequences. As the term is used herein, an amino acid or nucleic acid sequence is "homologous" to another sequence if the two sequences are substantially identical and the functional activity of the sequences is conserved (for example, both sequences function as or encode a FAE1 enzyme; as used herein, the term 'homologous' does not infer evolutionary relatedness). Nucleic acid sequences may also be homologous if they encode substantially identical amino acid sequences, even if the nucleic acid sequences are not themselves substantially identical, a circumstance that may for example arise as a result of the degeneracy of the genetic code.

Two amino acid or nucleic acid sequences are considered substantially identical if, when optimally aligned (with gaps permitted), they share at least about 50% sequence similarity or identity, or if the sequences share defined functional motifs. In alternative embodiments, sequence similarity in optimally aligned substantially identical sequences may be at least 60%, 70%, 80%, 90% or 95%. As used herein, a given percentage of homology between sequences denotes the degree of sequence identity in optimally aligned sequences.

Optimal alignment of sequences for comparisons of similarity may be automated using a variety of algorithms, such as the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math* 2: 482, the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444, and the computerized implementations of these algorithms (such as GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI, U.S.A.). Sequence similarity may also be determined using the BLAST algorithm, described in Altschul *et al.* (1990), *J. Mol. Biol.* 215:403-10 (using the published default settings). Software and instructions for performing BLAST analysis may be available through the National Center for Biotechnology Information in the United States (including the programs BLASTP, BLASTN, BLASTX, TBLASTN and TBLASTX that may be available through the internet at http://www.ncbi.nlm.nih.gov/). The BLAST algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database (reference) sequence. T is referred to as the neighborhood word score threshold. Initial neighborhood word hits act as seeds for initiating searches to find longer HSPs. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is halted when the following parameters are met: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program may use as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci. USA* 89: 10915-10919), a gap existence cost of 11, a per residue gap cost of 1, a lambda ratio of 0.85, alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands. One measure of the statistical similarity between two sequences using the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. In alternative embodiments of the invention, nucleotide or amino acid sequences are considered substantially identical if the smallest sum probability in a comparison of the test sequences is less than about 1, preferably less than about 0.1, more preferably less than

about 0.01, and most preferably less than about 0.001. In the PSI-BLAST implementation of the BLAST algorithm, an expect value for inclusion in PSI-BLAST iteration may be 0.001 (Altschul et al. (1997), Nucleic Acids Res. 25:3389-3402). Searching parameters may be varied to obtain potentially homologous sequences from database searches.

An alternative indication that two nucleic acid sequences are substantially identical is that the two sequences hybridize to each other under moderately stringent, or preferably stringent, conditions. Hybridization to filter-bound sequences under moderately stringent conditions may, for example, be performed in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65EC, and washing in 0.2 x SSC/0.1% SDS at 42EC (see Ausubel, *et al.* (eds), 1989, *Current Protocols in Molecular Biology*, Vol. 1, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3). Alternatively, hybridization to filter-bound sequences under stringent conditions may, for example, be performed in 0.5 M $NaHPO_4$, 7% SDS, 1 mM EDTA at 65EC, and washing in 0.1 x SSC/0.1% SDS at 68EC (see Ausubel, *et al.* (eds), 1989, *supra*). Hybridization conditions may be modified in accordance with known methods depending on the sequence of interest (see Tijssen, 1993, *Laboratory Techniques in Biochemistry and Molecular Biology -- Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York). Generally, stringent conditions are selected to be about 5EC lower than the thermal melting point for the specific sequence at a defined ionic strength and pH.

A *FAE1* promoter is any naturally occurring transcriptional regulatory region that mediates or modulates the expression of a plant FAE1 condensing enzyme. Plant FAE1 condensing enzymes are proteins that are homologous to known FAE1 condensing enzymes, such as those cloned and described in International Patent Publication WO 96/13582.

Heterologous DNA sequences may for example be introduced into a host cell by transformation. Such heterologous molecules may include sequences derived from the host cell species, which have been isolated and reintroduced into cells of the host species. Heterologous nucleic acid sequences may become integrated into a host cell genome, either as a result of the original transformation of the host cells, or as the result of subsequent recombination events. Transformation techniques that may be employed include plant cell membrane disruption by electroporation, microinjection and polyethylene glycol based transformation (such as are disclosed in Paszkowski *et al. EMBO J.* 3:2717 (1984); Fromm *et al., Proc. Natl. Acad. Sci. USA* 82:5824 (1985); Rogers *et al., Methods Enzymol.* 118:627

(1986); and in U.S. Patent Nos. 4,684,611; 4,801,540; 4,743,548 and 5,231,019), biolistic transformation such as DNA particle bombardment (for example as disclosed in Klein, *et al.*, *Nature* 327: 70 (1987); Gordon-Kamm, *et al.* "The Plant Cell" 2:603 (1990); and in U.S. Patent Nos. 4,945,050; 5,015,580; 5,149,655 and 5,466,587); *Agrobacterium*-mediated transformation methods (such as those disclosed in Horsch *et al. Science* 233: 496 (1984); Fraley *et al.*, *Proc. Nat'l Acad. Sci. USA* 80:4803 (1983); and U.S. Patent Nos. 4,940,838 and 5,464,763).

Standard methods are available for the preparation of constructs for use in identifying and characterizing transcriptional regulatory regions useful in various embodiments of the invention. General molecular techniques may for example be performed by procedures generally described by Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Stuhl K. (1995) Current Protocols in Molecular Biology, Vols 1, 2 and 3. Alternative equivalent methods or variations thereof may be used in accordance with the general knowledge of those skilled in this art and the functional requirements of the present invention.

In some aspects of the invention, transformed plant cells may be cultured to regenerate whole plants having a transformed genotype and displaying a desired phenotype, as for example modified by the expression of a heterologous protein mediated by a transcriptional regulatory region of the invention. A variety of plant culture techniques may be used to regenerate whole plants, such as are described in Gamborg and Phillips, "Plant Cell, Tissue and Organ Culture, Fundamental Methods", Springer Berlin, 1995); Evans *et al.* "Protoplasts Isolation and Culture", Handbook of Plant Cell Culture, Macmillian Publishing Company, New York, 1983; or Binding, "Regeneration of Plants, Plant Protoplasts", CRC Press, Boca Raton, 1985; or in Klee *et al.*, *Ann. Rev. of Plant Phys.* 38:467 (1987). A cell, tissue, organ, or organism into which has been introduced a foreign nucleic acid, is considered "transformed", "transfected", or "transgenic". A transgenic or transformed cell or organism also includes progeny of the cell or organism and progeny produced from a breeding program employing a transgenic plant as a parent in a cross and exhibiting an altered phenotype resulting from the presence of a recombinant nucleic acid construct. A transgenic plant is therefore a plant that has been transformed with a heterologous nucleic acid, or the progeny of such a plant that includes the transgene. The invention provides vectors, such as vectors for transforming plants or plant cells. The term "vector" in reference to nucleic acid molecule generally refers to a molecule that may be used to transfer a nucleic acid segment(s) from one cell to another. One of skill will recognize that after the nucleic acid is stably incorporated in transgenic plants and

confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques may be used, depending upon the species to be crossed.

In various embodiments, the invention comprises plants transformed with the nucleic acids of the invention. In some embodiments, such plants will exhibit altered fatty acid content in one or more tissues. These aspects of the invention relate to all higher plants, including monocots and dicots, such as species from the genera *Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonelia, Vigna, Citrus, Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Cichorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Heterocallis, Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browallia, Glycine, Lolium, Zea, Triticum, Sorghum, and Datura*. Such plants may include maize, wheat, rice, barley, soybean, beans, rapeseed, canola, alfalfa, flax, sunflower, cotton, clover, lettuce, tomato cucurbits, potato carrot, radish, pea lentils, cabbage, broccoli, brussel sprouts, peppers, apple, pear, peach, apricot, carnations and roses. More specifically, in alternative embodiments, plants for which the invention may be used in modifying fatty acid content include oil crops of the *Cruciferae* family: canola, rapeseed (*Brassica* spp.), crambe (*Crambe* spp.), honesty (*Lunaria* spp.) lesquerella (*Lesquerela* spp.), and others; the *Compositae* family: sunflower (*Helianthus* spp.), safflower (*Carthamus* spp.), niger (*Guizotia* spp.) and others; the *Palmae* family: palm (*Elaeis* spp.), coconut (*Cocos* spp.) and others; the *Leguminosae* family: peanut (*Arachis* spp.), soybean (*Glycine* spp.) and others; and plants of other families such as maize (*Zea* spp.), cotton (*Gossypium* sp.), jojoba (*Simonasia* sp.), flax (*Linum* sp.), sesame (*Sesamum* spp.), castor bean (*Ricinus* spp.), olive (*Olea* spp.), poppy (*Papaver* spp.), spurge (*Euphorbia*, spp.), meadowfoam (*Limnanthes* spp.), mustard (*Sinapis* spp.) and cuphea (*Cuphea* spp.).

Nucleic acids of the invention may also be used as a plant breeding tool, as molecular markers to aid in plant breeding programs. Such techniques would include using the gene itself as a molecular probe or using the DNA sequence to design PCR primers to use PCR based screening techniques in plant breeding programs.

Deletion or insertion constructs may be useful for domain mapping to determine the functional domains or motifs of a transcriptional regulatory region derived from a *FAE1* gene. An aspect of the invention is the construction and testing of such constructs, as described below for the 5' deletion construct of the *A. thaliana FAE1* 5' region. One aspect of the

invention comprises transcriptional regulatory regions that are derived from functionally important regions of a *FAE1* promoter. As outlined above, the functionally important regions of a *FAE1* promoter may be determined through routine assays. Alternatively, randomly selected portions of a a *FAE1* promoter may be selected for use in routine assays to determine whether the selected region is capable of functioning as a transcriptional regulatory region in the context of the present invention. In various embodiments, regions of the *Arabidopsis thaliana, Brassica napus* or *Lunaria annua* promoters may be used. For example, the following motifs in the *A.t. FAE1* promoter may be used alone or in combination in novel transcriptional regulatory regions (see Figure 4): the CE-like elements (CE1 and CE3), the RY repeat motif, the G-boxes (G-box1 and G-box2), the A-300 box, the EM1 ABA box, or the CTATTTTG element. Constructs of the invention comprising such motifs, deletions or insertions may be assayed for activity as transcriptional regulatory regions of the invention by testing for strong seed-specific activity providing expression of a sequence of interest (such as a reporter sequence) before the torpedo stage and persisting throughout embryo development, in accordance with standard testing methods that may be adapted from the methods disclosed herein.

Alternative embodiments of the transcriptional regulatory regions of the invention may be identified using information available through NCBI databases at http://www.ncbi.nih.gov.

In various embodiments, transcriptional regulatory regions derived from plant *FAE1* genes are shown to be capable of directing expression of desired genes at an early stage of development in a seed-specific manner in disparate plant species. In particular embodiments, the transcriptional regulatory regions of the invention may be used in a wide variety of dicotyledonous plants for modification of the seed phenotype. For example, new seed phenotypes may include:

(1) altered seed fatty acid composition or seed oil composition and accumulation
(2) altered seed protein or carbohydrate composition or accumulation
(3) enhanced production of desirable endogenous seed products
(4) suppression of production of undesirable gene products using antisense, co-suppression or ribozyme technologies
(5) production of novel recombinant proteins for pharmaceutical, industrial or nutritional purposes

Isolation of a seed-specific promoter from *A. thaliana*

Using the sequence information of the *A. thaliana* genome sequencing project, synthetic oligonucletide primers were designed to amplify the *FAE1* 5' untranslated region, to isolate it by PCR. As shown in Figure 1, the upstream primer 5'-CTAGTAGATTGGTTGGTTGGTTTCC-3' (AtproFW) in combination with the downstream primer 5'-TGCTCTGTTTGTGTCGGAAAATAATGG-3' (AtproRV) were used, and resulted in the synthesis of a fragment of the correct size (934 bp). The amplified product was subcloned in the *Hin*cII site of the plasmid pT7T3-18U (Pharmacia) to produce plasmid pT7T3-18U/proFAE900, followed by complete sequence determination of both strands to verify the fragment identity. A BLAST search of the *A. thaliana* Database identified a single BAC clone T4L20 (GenBank ATF10M6) 125,179 bp long, which contains the complete *FAE1* gene.

Functional analysis of the *FAE1* 5' upstream region

5' upstream fragments of the *FAE1* gene were shown to confer seed-specific and temporally regulated gene expression in plants. A translational fusion was made between the *FAE1* 5' region and the coding region of the reporter gene β-glucuronidase (GUS). The chimeric gene (pFAE900-GUS or pFAE400-GUS) was transferred into *Arabidopsis* and tobacco and GUS activity was monitored in various tissue of transgenic plants.

Construction of the vectors pFAE900-GUS and pFAE400-GUS, and transformation of *Arabidopsis* and tobacco, was as follows. The insert was cleaved out of pT7T3-18U vector with *Hin*dIII and *Xba*I and directionally subcloned into the corresponding sites of the binary Ti plasmid pBI101 (Clontech), which contains a promoterless GUS gene (Jefferson et al. 1987), to obtain the vector pFAE900-GUS. Another construct, pFAE400-GUS, containing only 393 bp of the 5' *FAE1* region directly upstream of the ATG initiation codon fused to the GUS coding sequence was also generated. For that, the pT7T3-18U/proFAE900 vector was digested with *BglII* and *PstI*, the sticky ends were filled in using T4 DNA polymerase, followed by re-ligation to obtain pT7T3-18U/proFAE400. The 393 bp 5' *FAE1* upstream fragment was then excised with *Hin*dIII and *Xba*I and cloned into the binary vector pBI101 to obtain the plasmid pFAE400-GUS. The pFAE400-GUS and pFAE900-GUS fusion constructs in pBI101 were introduced into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) by heat-shock and selected for resistance to kanamycin (50 μg/ml). *A. thaliana* (L.) Heynh. ecotype Columbia was transformed with the pFAE400-GUS and pFAE900-GUS constructs using floral dip method (Clough and Bent, 1998). Screening for transformed seed

was done on 50μg/mL kanamycin as described previously (Katavic et al., 1994). Approximately 100 transgenic lines were generated for each construct.

For transformation of tobacco, *A. tumefaciens* harbouring the pFAE900-GUS construct was co-cultivated with leaf pieces of *Nicotiana tabacum* SR1 and transformants were selected with kanamycin (100μg/mL) on solid medium (Lee and Douglas, 1996).

Histochemical localization of GUS activity and analysis of transgenic plants was as follows. Tissue sections were placed in 100 mM $NaPO_4$ (pH7) and 1 mM spermidine for 15 min, then incubated at 37° C in 0.5 $K_3[Fe(CN)_6]$, 0.01 % Triton X-100, 1mM EDTA, 10 mM β-mercaptoethanol, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM $NaPO_4$ (pH7), until a blue color appeared (after approximately 1 hr). Following incubation with the substrate, chlorophyll was removed from the sections using a graded ethanol series.

Using this assay, five independent transgenic *Arabidopsis* lines were examined for the embryo-specific expression of the GUS gene. In addition, leaf, stem and siliques were histochemically stained for β-glucuronidase activity. The results indicate that the reporter gene fused to the transcriptional regulatory region of the invention is not expressed in vegetative tissues, whereas it is highly expressed in developing seeds (embryos). Both the 934 bp and the 393 bp transcriptional regulatory regions derived from the *A.t. FAE1* gene caused the appearance of GUS activity by the torpedo stage embryo (6 days after flowering). GUS activity in all five lines persisted throughout subsequent embryo development.

Leaves, stems, pods and seeds of three regenerated tobacco lines transformed with the pFAE900-GUS construct were also assayed for β-glucuronidase activity. The results obtained indicate that the 934 bp *FAE1* promoter fragment contains sufficient information to direct seed-specific expression of a reporter gene in transgenic tobacco. Thus the transcriptional regulatory regions of the invention may be used for seed-specific expression of foreign genes in transgenic plants.

The *in vivo* activity of a *FAE1* promoter of the invention was compared to the activity of the napin promoter by expressing the castor bean hydroxylase gene *FAH12* (Broun and Somerville, 1997) behind either the *FAE1*-promoter (a transcriptional regulatory region of approximately 1 kb) or the napin promoter in an Arabidopsis *fad2/fae1* double mutant. This mutant accumulates as a proportion of fatty acids about 85% of the 18:1 acyl group, which is the substrate for the hydroxylase. The levels of hydroxylated fatty acids accumulating in a large number of independent transgenic lines were used to estimate the relative strength of

each promoter. As shown in Figure 6, the two populations of transgenic plants accumulated levels of hydroxylated fatty acids, ranging from 0.2% to about 11-12% of total fatty acids, with the levels being on average slightly higher in *FAE1-FAH12* lines. Similarly, the best *FAE1-FAH12* plant accumulated just over 12% of hydroxylated fatty acids (w/w of total FAs), whereas the best *napin-FAH12* plant produced 10.8% of hydroxylated fatty acids (w/w of total FAs). These results indicate that the FAE1 promoter is highly active in transgenic *Arabidopsis* and that its *in vivo* activity may be superior to napin in *Arabidopsis* seeds.

Sequence elements or motifs that confer both tissue specificity and developmental regulation of transcription reside within 393 bp of the AUG translation initiation codon in the *A.t. FAE1* gene. The seed-specific expression conferred by the transcriptional regulatory regions of the invention is independent of the native terminator of the *FAE1* gene 3' end. For example, in the exemplified constructs disclosed herein, a terminator derived from the *Agrobacterium* nopaline synthase gene was used.

Lunaria annua and *Brassica napus FAE1* 5' regulatory regions

Two sequences originating from *B. napus* and *L. annua* were isolated and characterized to demonstrate that regulatory regions conferring seed-specific transcription early in embryo development can also be found upstream of other plant *FAE1* genes. Sequences were cloned using the technique of polymerase chain reaction (PCR) walking on uncloned plant genomic DNA (Devic et al., 1997). Approximately 5 µg of genomic DNA from 1 g of fresh tissue was used for the construction of 5 different libraries by digesting DNA with a series of enzymes that produce blunt end fragments to which special adaptors are ligated. The adaptor molecules consist of a long upper strand, which contains successive sequences common to the adaptor primers, AP1 and AP2, annealed at its 3' end to a shorter strand lacking the AP1 sequence. However, this short strand posseses an amine group at its 3' end to prevent filling in by the DNA polymerses during the first PCR amplification step and generation of the AP1 binding site. This suppression PCR effect prevents exponential amplification of molecules containing the adaptor at each end, and the adaptor primer binding sites are only produced when a strand complementary to the upper strand of the adaptor is synthesized by extension from a gene specific primer. The first PCR reaction is performed using an adaptor primer AP1 and a gene specific primer. An aliquot of the first PCR product is used a template in a second PCR amplification using the nested gene specific primer and AP2.

In order to isolate the regulatory regions upstream of the *B. napus FAE1* coding sequence, genomic DNA was prepared from developing leaves and digested with 5 blunt-end

cutting restriction enzymes (*DraI, EcoRV, HpaI, PvuII and ScaI*) to generate a series of DNA libraries. After ligation of adapter molecules, individual libraries were used as templates in a two step PCR. In the first PCR amplification using the AP1 primer 5'-GGATCCTAATACGACTCACTATAGGGC-3' and the *FAE1* gene specific primer 5'-AAAGAGTGGAGCGATGGTTATGAGG-3' (Bnwalk1), multiple DNA fragments were amplified from all five library templates. After a second round of PCR, using the AP2 primer 5'-CTATAGGGCTCGAGCGGC-3' and the nested *FAE1* specific primer 5'-CGGAAAGAAGCAAAGGTTGAAAAGG-3' (Bnwalk2), the longest single fragment of 1.6 kb was obtained from the *HpaI* library template. This fragment was inserted into the pCR2.1 plasmid (Invitrogen) and sequenced. The sequence is shown in Figure 2.

For the PCR walking experiment to isolate the *L. annua* 5' regulatory region, in adition to the standard AP1 and AP2 primers, the following *FAE1* specific primers were used: 5'-GATCGTTTGTGGTAAGACGAGAGC-3' (Lawalk1) and 5'-GTCAGTGGGAAGAAACAGAGGTTG-3' (Lawalk2). In the first PCR reaction, the *DraI, EcoRV, PvuII, ScaI* and *SspI* library templates were used. In a second PCR amplification the longest single fragment 1.1 kb in length was synthesized using the *EcoRV* library template. This fragment was inserted into the *HincII* site of the pT7T3-18U vector (Promega), sequenced on both strands and analyzed (Figure 3).

Using the sequence data obtained for the 5' regulatory regions generated by PCR walking, specific primers were generated for the amplification of the *L. annua* and *B. napus FAE1* promoter fragments. For the PCR-amplification of *B. napus* promoter fragment the upstream primer was 5'-CTGACTTCACCAAAGAAACAACTCG-3' (BnproFW) in combination with the downstream primer 5'-CGGAATTCCGTTTTTTTTTTTAGGCG-3' (BnproRV). The synthesized fragment was ligated into the *SmaI* site of pGEM-7Zf (Promega), then excised with *XbaI/BamHI* and cloned into the equivalent sites of the pBI101 binary vector (Clontech). *L. annua* 5' regulatory region was amplified using the 5'-CAGCTTAACCGGTAAAATTGGCC-3' (LaproFW) upstream primer together with the 5'-TGTTCAGTTTTGTGTCGGAGAGG-3' (LaproRV) downstream primer and inserted in the *HincII* site of pT7T3-18U (Promega) plasmid. In order to clone the *L. annua* promoter fragment into the pBI101 binary vector, an *XbaI* site was added by subcloning the *PstI/KpnI* fragment released from the pT7T3-18U vector into pBluescript II KS+ (Stratagene). The fragment was then excised and cloned in the *XbaI* site of the pBI101 vector.

The resulting vectors pBnFAE1-GUS and pLaFAE1-GUS in pBI101 were then introduced into *A. tumefaciens* strain GV3101 by heat-shock, and used to transform *Arabidopsis* as described above. Transformants were selected on agar-solidified medium containing kanamycin (50 μg/ml). More than 100 transformants were generated for each construct. The activity of the *L. annua* and *B. napus FAE1* promoters was determined by GUS expression assays on the developing seeds and also on non-reproductive plant tissues as controls. Consistent seed-specific GUS expression was obtained for both promoter constructs in independent transgenic lines. In contrast, there was no detectable GUS activity in leaf, stem and silique samples.

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WHAT IS CLAIMED IS:

1. A recombinant nucleic acid molecule comprising a heterologous promoter sequence operably linked to a nucleic acid sequence, wherein the promoter sequence comprises a transcriptional regulatory region capable of mediating seed-specific expression in *Arabidopsis* wherein the transcriptional regulatory region:

 (a) is obtainable from a 5' region of a plant *FAE1* gene; or

 (b) hybridizes under stringent conditions to the 5' region of the plant *FAE1* gene; or

 (c) is at least 70% identical when optimally aligned to the 5' region of the plant *FAE1* gene.

2. The recombinant nucleic acid of claim 1 wherein the 5' region of the plant *FAE1* gene comprises (5' to 3'):

AGA TCTAAGAACA CACATTCCCT CAAATTTTAA TGCACATGTA
ATCATAGTTT AGCACAATTC AAAAATAATG TAGTATTAAA GACAGAAATT
TGTAGACTTT TTTTTGGCGT TAAAGGAAGA CTAAGTTTAT ACGTACATTT
TATTTTAAGT GGAAAACCGA AATTTTCCAT CGAAATATAT GAATTTAGTA
TATATATTTC TGCAATGTAC TATTTTGCTA TTTTGGCAAC TTTCAGTGGA
CTACTACTTT ATTACAATGT GTATGGATGC ATGAGTTTGA GTATACACAT
GTCTAAATGC ATGCTTTGCA AAACGTAACG GACCACAAAA GAGGATCCAT
GCAAATACAT CTCATAGCTT CCTCCATTAT TTTCCGACAC AAACAGAGCA.

3. The recombinant nucleic acid of claim 1 wherein the 5' region of the plant *FAE1* gene comprises (5' to 3'):

AAGGCTTACC CTATTAGTTG AAAGTTGAAA CTTTGTTCCC TACTCAATTC
CTAGTTGTGT AAATGTATGT ATATGTAATG CGTATAAAAC GTAGTACTTA
AATGACTAGG AGTGGTTCTT GAGACCGATG AGAGATGGGA GCAGAACTAA
AGATGATGAC ATAATTAAGA ACGAATTTGA AAGGCTCTTA GGTTTGAATC
CTATTCGAGA ATGTTTTTGT CAAAGATAGT GGCGATTTTG AACCAAAGAA
AACATTTAAA AAATCAGTAT CCGGTTACGT TCATGCAAAT AGAAAGTGGT
CTAGGATCTG ATTGTAATTT TAGACTTAAA GAGTCTCTTA AGATTCAATC
CTGGCTGTGT ACAAAACTAC AAATAATATA TTTTAGACTA TTTGGCCTTA
ACTAAACTTC CACTCATTAT TTACTGAGGT TAGAGAATAG ACTTGCGAAT

AAACACATTC CCGAGAAATA CTCATGATCC CATAATTAGT CAGAGGGTAT
GCCAATCAGA TCTAAGAACA CACATTCCCT CAAATTTTAA TGCACATGTA
ATCATAGTTT AGCACAATTC AAAAATAATG TAGTATTAAA GACAGAAATT
TGTAGACTTT TTTTTGGCGT TAAAGGAAGA CTAAGTTTAT ACGTACATTT
TATTTTAAGT GGAAAACCGA AATTTTCCAT CGAAATATAT GAATTTAGTA
TATATATTTC TGCAATGTAC TATTTTGCTA TTTTGGCAAC TTTCAGTGGA
CTACTACTTT ATTACAATGT GTATGGATGC ATGAGTTTGA GTATACACAT
GTCTAAATGC ATGCTTTGCA AAACGTAACG GACCACAAAA GAGGATCCAT
GCAAATACAT CTCATAGCTT CCTCCATTAT TTTCCGACAC AAACAGAGCA.

4. The recombinant nucleic acid of claim 1 wherein the 5' region of the plant *FAE1* gene comprises (5' to 3'):

CTGACTTC ACCAAAGAAA CAACTCGAGT CGTTATCCAT
CTCCTCATAA CCATCGCTCC ACTCTTTGCC TTCACCGTTT TCGGTTCGGT
TCTCTACATC GCAACCCGGC CCAAACCGGT TTACCTCGTT GAGTACTCAT
GCTACCTTCC ACCAACGCAT TGTAGATCAA GTATCTCCAA GGTCATGGAT
ATCTTTTATC AAGTAAGAAA AGCTGATCCT TCTCGGAACG GCACGTGCGA
TGACTCGTCG TGGCTTGACT TCTTGAGGAA GATTCAAGAA CGTTCAGGTC
TAGGCGATGA AACTCACGGG CCCGAGGGGC TGCTTCAGGT CCCTCCCCGG
AAGACTTTTG CGGCGGCGCG TGAAGAGACG GAGCAAGTTA TCATTGGTGC
GCTAGAAAAT CTATTCAAGA ACACCAACGT TAACCCTAAA GATATAGGTA
TACTTGTGGT GAACTCAAGC ATGTTTAATC CAACTCCATC GCTCTCCGCG
ATGGTCGTTA ACACTTTCAA GCTCCGAAGC AACGTAAGAA GCTTTAACCT
TGGTGGCATG GGTTGTAGTG CCGGCGTTAT AGCCATTGAT CTAGCAAAGG
ACTTGTTGCA TGTCCATAAA AATACGTATG CTCTTGTGGT GAGCACAGAG
AACATCACTT ATAACATTTA CGCTGGTGAT AATAGGTCCA TGATGGTTTC
AAATTGCTTG TTCCGTGTTG GTGGGGCCGC TATTTTGCTC TCCAACAAGC
CTGGAGATCG TAGACGGTCC AAGTACGAGC TAGTTCACAC GGTTCGAACG
CATACCGGAG CTGACGACAA GTCTTTTCGT TGCGTGCAAC AAGGAGACGA
TGAGAACGGC AAAATCGGAG TGAGTTTGTC CAAGGACATA ACCGATGTTG
CTGGTCGAAC GGTTAAGAAA AACATAGCAA CGTTGGGTCC GTTGATTCTT
CCGTTAAGCG AGAAACTTCT TTTTTTCGTT ACCTTCATGG GCAAGAAACT
TTTCAAAGAT AAAATCAAAC ATTACTACGT CCCGGATTTC AAACTTGCTA
TTGACCATTT TTGTATACAT GCCGGAGGCA GAGCCGTGAT TGATGTGCTA
GAGAAGAACC TAGCCCTAGC ACCGATCGAT GTAGAGGCAT CAAGATCAAC
GTTACATAGA TTTGGAAACA CTTCATCTAG CTCAATATGG TATGAGTTGG

CATACATAGA AGCAAAAGGA AGGATGAAGA AAGGTAATAA AGTTTGGCAG
ATTGCTTTAG GGTCAGGCTT TAAGTGTAAC AGTGCAGTTT GGGTGGCTCT
AAACAATGTC AAAGCTTCGA CAAATAGTCC TTGGGAACAC TGCATCGACA
GATACCCGGT CAAAATTGAT TCTGATTCAG GTAAGTCAGA GACTCGTGTC
CAAAACGGTC GGTCCTAATA AACGATGTTT GCTCTCTTTC GTTTCTTTTT
ATTTGTTATA ATAATTTGAT GGCTACGATG TTTCTCTTGT TTGTTATGAA
TAAAGAATGC AATGGTGTTC TAGTATTTGA TTGTTTTACA TGTATGTATC
TCTTATTTAC ATGAAATTTT TAAACGCCTA AAAAAAAAAA CGGAATTCCG.

5. The recombinant nucleic acid of claim 1 wherein the 5' region of the plant *FAE1* gene comprises (5' to 3'):

CAGCTTAAC CGGTAAAATT
GGCCTGTACA TATATTTACC ACTGAGTAAA GACATCAGTT AATGATTTGT
TGTTACTCAA TTGGGCTAAG TGTATTATTA TATGTGTTGT ATATAATAAA
GGTAGAACGT AAATTTACTA AGAATGTGTT TTTCCAATGT GATTGCTCTT
TGGCCTCTTA GGTTTGAATC CTACTCGAGA AGACTAATTT TAATTTACTG
GCAAAAATAG AAATCAATTT ATAAGTGTTT AAACAAATCG ATGGTATAAC
TGATTAGTGA TCACTCTTAG GTTTTGATCC AACTCGAGTA TTGAGTATTG
AACGCTTTTT TTAAATAAAA TCTTGATTTT TAAATTGGTT TTTTGAGTAA
AAAAGTTCTT AATATTTTCT CTTTGTTTTA ATGGGTTTGT TTTGCATTTT
ATAAGCTTAA TTTTTCTAAT TTAATATTTT ATCTATCATC GTCCGTAAAG
TTTTATTTGG CACAAACTTG TTTTACTTTT CTACCTTATA ATTTGGGAAC
TGGTTGAGTC AAAGCGTACC GGACAAATAT GTTTTATATT CTTATTTAAG
AATTAACACT CATCTCATAA TTAGTCAGAG GCTAGGGAGA TTCAGCCAAT
CAATGCTAAC AACAAAATTC TCTTAATGAT CTAACGATGC TATTTAATAT
TCGGATCAGT ATTCTTAAAT AAGAATATAA AACTAATTCA ATAGTTACAG
ATAAAAACTT ATATAGACTT TTTTATTTGG AATATAAAAG TATCAATATA
TTATAGACAA TATTTATAAC GTTAAAAATA CAATATTTAT ATTTTTTATA
TATTTATTTC AAATTGAAAA GCATTACTTC TATCGAAATG AATTTTAGTA
TATTAATTAA TATTTTTTTA ATCGGACTAC TTTCCTATTT TGGCACCTTT
CATCTGACTA CTAATTTATT TCAATGTGTA TGCATGCATG AGCATGAGTA
ATACACATGT CTATATAAAT GCATGTAAAA CGTAACGGAC CACAAAAGTG
GATCCATACA AATACATCTC ATCGCACCCT CTCCGACACA AAACTGAACA.

6. The recombinant nucleic acid of claim 1 wherein the promoter sequence is selected from the group consisting of *Arabidopsis thaliana*, *Lunaria annua* and *Brassica napus* *FAE1* promoter sequences.

7. The recombinant nucleic acid of any one of claims 1 through 6, wherein the transcriptional regulatory region is at least 70% identical when optimally aligned to the 5' region of the plant *FAE1* gene..

8. The recombinant nucleic acid of claim 1 wherein the transcriptional regulatory region comprises (5' to 3'):

AGA TCTAAGAACA CACATTCCCT CAAATTTTAA TGCACATGTA
ATCATAGTTT AGCACAATTC AAAAATAATG TAGTATTAAA GACAGAAATT
TGTAGACTTT TTTTTGGCGT TAAAGGAAGA CTAAGTTTAT ACGTACATTT
TATTTTAAGT GGAAAACCGA AATTTTCCAT CGAAATATAT GAATTTAGTA
TATATATTTC TGCAATGTAC TATTTTGCTA TTTTGGCAAC TTTCAGTGGA
CTACTACTTT ATTACAATGT GTATGGATGC ATGAGTTTGA GTATACACAT
GTCTAAATGC ATGCTTTGCA AAACGTAACG GACCACAAAA GAGGATCCAT
GCAAATACAT CTCATAGCTT CCTCCATTAT TTTCCGACAC AAACAGAGCA.

9. The recombinant nucleic acid of claim 1 wherein the transcriptional regulatory region comprises (5' to 3'):

AAGGCTTACC CTATTAGTTG AAAGTTGAAA CTTTGTTCCC TACTCAATTC
CTAGTTGTGT AAATGTATGT ATATGTAATG CGTATAAAAC GTAGTACTTA
AATGACTAGG AGTGGTTCTT GAGACCGATG AGAGATGGGA GCAGAACTAA
AGATGATGAC ATAATTAAGA ACGAATTTGA AAGGCTCTTA GGTTTGAATC
CTATTCGAGA ATGTTTTTGT CAAAGATAGT GGCGATTTTG AACCAAAGAA
AACATTTAAA AAATCAGTAT CCGGTTACGT TCATGCAAAT AGAAAGTGGT
CTAGGATCTG ATTGTAATTT TAGACTTAAA GAGTCTCTTA AGATTCAATC
CTGGCTGTGT ACAAAACTAC AAATAATATA TTTTAGACTA TTTGGCCTTA
ACTAAACTTC CACTCATTAT TTACTGAGGT TAGAGAATAG ACTTGCGAAT
AAACACATTC CCGAGAAATA CTCATGATCC CATAATTAGT CAGAGGGTAT
GCCAATCAGA TCTAAGAACA CACATTCCCT CAAATTTTAA TGCACATGTA
ATCATAGTTT AGCACAATTC AAAAATAATG TAGTATTAAA GACAGAAATT
TGTAGACTTT TTTTTGGCGT TAAAGGAAGA CTAAGTTTAT ACGTACATTT

TATTTTAAGT GGAAAACCGA AATTTTCCAT CGAAATATAT GAATTTAGTA
TATATATTTC TGCAATGTAC TATTTTGCTA TTTTGGCAAC TTTCAGTGGA
CTACTACTTT ATTACAATGT GTATGGATGC ATGAGTTTGA GTATACACAT
GTCTAAATGC ATGCTTTGCA AAACGTAACG GACCACAAAA GAGGATCCAT
GCAAATACAT CTCATAGCTT CCTCCATTAT TTTCCGACAC AAACAGAGCA.

10. The recombinant nucleic acid of claim 1 wherein the transcriptional regulatory region comprises (5' to 3'):

CTGACTTC ACCAAAGAAA CAACTCGAGT CGTTATCCAT
CTCCTCATAA CCATCGCTCC ACTCTTTGCC TTCACCGTTT TCGGTTCGGT
TCTCTACATC GCAACCCGGC CCAAACCGGT TTACCTCGTT GAGTACTCAT
GCTACCTTCC ACCAACGCAT TGTAGATCAA GTATCTCCAA GGTCATGGAT
ATCTTTTATC AAGTAAGAAA AGCTGATCCT TCTCGGAACG GCACGTGCGA
TGACTCGTCG TGGCTTGACT TCTTGAGGAA GATTCAAGAA CGTTCAGGTC
TAGGCGATGA AACTCACGGG CCCGAGGGGC TGCTTCAGGT CCCTCCCCGG
AAGACTTTTG CGGCGGCGCG TGAAGAGACG GAGCAAGTTA TCATTGGTGC
GCTAGAAAAT CTATTCAAGA ACACCAACGT TAACCCTAAA GATATAGGTA
TACTTGTGGT GAACTCAAGC ATGTTTAATC CAACTCCATC GCTCTCCGCG
ATGGTCGTTA ACACTTTCAA GCTCCGAAGC AACGTAAGAA GCTTTAACCT
TGGTGGCATG GGTTGTAGTG CCGGCGTTAT AGCCATTGAT CTAGCAAAGG
ACTTGTTGCA TGTCCATAAA AATACGTATG CTCTTGTGGT GAGCACAGAG
AACATCACTT ATAACATTTA CGCTGGTGAT AATAGGTCCA TGATGGTTTC
AAATTGCTTG TTCCGTGTTC GTGGGGCCGC TATTTTGCTC TCCAACAAGC
CTGGAGATCG TAGACGGTCC AAGTACGAGC TAGTTCACAC GGTTCGAACG
CATACCGGAG CTGACGACAA GTCTTTTCGT TGCGTGCAAC AAGGAGACGA
TGAGAACGGC AAAATCGGAG TGAGTTTGTC CAAGGACATA ACCGATGTTG
CTGGTCGAAC GGTTAAGAAA AACATAGCAA CGTTGGGTCC GTTGATTCTT
CCGTTAAGCG AGAAACTTCT TTTTTTCGTT ACCTTCATGG GCAAGAAACT
TTTCAAAGAT AAAATCAAAC ATTACTACGT CCCGGATTTC AAACTTGCTA
TTGACCATTT TTGTATACAT GCCGGAGGCA GAGCCGTGAT TGATGTGCTA
GAGAAGAACC TAGCCCTAGC ACCGATCGAT GTAGAGGCAT CAAGATCAAC
GTTACATAGA TTTGGAAACA CTTCATCTAG CTCAATATGG TATGAGTTGG
CATACATAGA AGCAAAAGGA AGGATGAAGA AAGGTAATAA AGTTTGGCAG
ATTGCTTTAG GGTCAGGCTT TAAGTGTAAC AGTGCAGTTT GGGTGGCTCT
AAACAATGTC AAAGCTTCGA CAAATAGTCC TTGGGAACAC TGCATCGACA

GATACCCGGT CAAAATTGAT TCTGATTCAG GTAAGTCAGA GACTCGTGTC
CAAAACGGTC GGTCCTAATA AACGATGTTT GCTCTCTTTC GTTTCTTTTT
ATTTGTTATA ATAATTTGAT GGCTACGATG TTTCTCTTGT TTGTTATGAA
TAAAGAATGC AATGGTGTTC TAGTATTTGA TTGTTTTACA TGTATGTATC
TCTTATTTAC ATGAAATTTT TAAACGCCTA AAAAAAAAAA CGGAATTCCG.

11. The recombinant nucleic acid of claim 1 wherein the transcriptional regulatory region comprises (5' to 3'):

CAGCTTAAC CGGTAAAATT
GGCCTGTACA TATATTTACC ACTGAGTAAA GACATCAGTT AATGATTTGT
TGTTACTCAA TTGGGCTAAG TGTATTATTA TATGTGTTGT ATATAATAAA
GGTAGAACGT AAATTTACTA AGAATGTGTT TTTCCAATGT GATTGCTCTT
TGGCCTCTTA GGTTTGAATC CTACTCGAGA AGACTAATTT TAATTTACTG
GCAAAAATAG AAATCAATTT ATAAGTGTTT AAACAAATCG ATGGTATAAC
TGATTAGTGA TCACTCTTAG GTTTTGATCC AACTCGAGTA TTGAGTATTG
AACGCTTTTT TTAAATAAAA TCTTGATTTT TAAATTGGTT TTTTGAGTAA
AAAAGTTCTT AATATTTTCT CTTTGTTTTA ATGGGTTTGT TTTGCATTTT
ATAAGCTTAA TTTTTCTAAT TTAATATTTT ATCTATCATC GTCCGTAAAG
TTTTATTTGG CACAAACTTG TTTTACTTTT CTACCTTATA ATTTGGGAAC
TGGTTGAGTC AAAGCGTACC GGACAAATAT GTTTTATATT CTTATTTAAG
AATTAACACT CATCTCATAA TTAGTCAGAG GCTAGGGAGA TTCAGCCAAT
CAATGCTAAC AACAAAATTC TCTTAATGAT CTAACGATGC TATTTAATAT
TCGGATCAGT ATTCTTAAAT AAGAATATAA AACTAATTCA ATAGTTACAG
ATAAAAACTT ATATAGACTT TTTTATTTGG AATATAAAAG TATCAATATA
TTATAGACAA TATTTATAAC GTTAAAAATA CAATATTTAT ATTTTTTATA
TATTTATTTC AAATTGAAAA GCATTACTTC TATCGAAATG AATTTTAGTA
TATTAATTAA TATTTTTTTA ATCGGACTAC TTTCCTATTT TGGCACCTTT
CATCTGACTA CTAATTTATT TCAATGTGTA TGCATGCATG AGCATGAGTA
ATACACATGT CTATATAAAT GCATGTAAAA CGTAACGGAC CACAAAAGTG
GATCCATACA AATACATCTC ATCGCACCCT CTCCGACACA AAACTGAACA.

12. The recombinant nucleic acid of any one of claims 1 through 11 wherein the nucleic acid sequence encodes a translatable mRNA.

13. The recombinant nucleic acid of claim 12 wherein the nucleic acid sequence encodes an enzyme involved in lipid metabolism.

14. The recombinant nucleic acid of any one of claims 1 through 13, further comprising a transcription termination region operably linked to the nucleic acid sequence.

15. A host cell comprising the recombinant nucleic acid of any one of claims 1 through 14.

16. The host cell of claim 15, wherein the host cell is of a dicotyledonous plant species.

17. A plant comprising the recombinant nucleic acid of any one of claims 1 through 14.

18. The plant of claim 17, wherein the plant is of a dicotyledonous plant species.

19. A method of altering the phenotype of a seed comprising:

a) transforming a seed-bearing plant, or a progenitor of the seed-bearing plant, with a vector comprising the nucleic acid of any one of claims 1 through 14;

b) growing the seed-bearing plant to obtain seed under conditions wherein the nucleic acid sequence is expressed during embryogenesis under the control of the transcriptional regulatory region to alter the phenotype of the seed.

20. A method of transforming a plant cell comprising transforming the plant cell with the recombinant nucleic acid of any one of claims 1 through 14.

Figure 1. ***Arabidopsis thaliana FAE1*** **promoter:**
(Length: 934 bp)

```
-950       ACTCA TAAAAACTAG TAGATTGGTT GGTTGGTTTC CATGTACCAG
                                        AtproFW →
-900 AAGGCTTACC CTATTAGTTG AAAGTTGAAA CTTTGTTCCC TACTCAATTC
-850 CTAGTTGTGT AAATGTATGT ATATGTAATG CGTATAAAAC GTAGTACTTA
-800 AATGACTAGG AGTGGTTCTT GAGACCGATG AGAGATGGGA GCAGAACTAA
-750 AGATGATGAC ATAATTAAGA ACGAATTTGA AAGGCTCTTA GGTTTGAATC
-700 CTATTCGAGA ATGTTTTTGT CAAAGATAGT GGCGATTTTG AACCAAAGAA
-650 AACATTTAAA AAATCAGTAT CCGGTTACGT TCATGCAAAT AGAAAGTGGT
-600 CTAGGATCTG ATTGTAATTT TAGACTTAAA GAGTCTCTTA AGATTCAATC
-550 CTGGCTGTGT ACAAAACTAC AAATAATATA TTTTAGACTA TTTGGCCTTA
-500 ACTAAACTTC CACTCATTAT TTACTGAGGT TAGAGAATAG ACTTGCGAAT
-450 AAACACATTC CCGAGAAATA CTCATGATCC CATAATTAGT CAGAGGGTAT
-400 GCCAATCAGA TCTAAGAACA CACATTCCCT CAAATTTTAA TGCACATGTA
-350 ATCATAGTTT AGCACAATTC AAAAATAATG TAGTATTAAA GACAGAAATT
-300 TGTAGACTTT TTTTTGGCGT TAAAGGAAGA CTAAGTTTAT ACGTACATTT
-250 TATTTTAAGT GGAAAACCGA AATTTTCCAT CGAAATATAT GAATTTAGTA
-200 TATATATTTC TGCAATGTAC TATTTTGCTA TTTTGGCAAC TTTCAGTGGA
-150 CTACTACTTT ATTACAATGT GTATGGATGC ATGAGTTTGA GTATACACAT
-100 GTCTAAATGC ATGCTTTGCA AAACGTAACG GACCACAAAA GAGGATCCAT
 -50 GCAAATACAT CTCATAGCTT CCTCCATTAT TTTCCGACAC AAACAGAGCA
                                  ← AtproRV
   1 ATGACGTCCG TTAACGTTAA GCTCCTT
```

Figure 2. ***Brassica napus FAE1*** **promoter:**
(Length: 1588 bp)

```
-1600 GGTTGGGCAA ATCTGACTTC ACCAAAGAAA CAACTCGAGT CGTTATCCAT
                                  BnproFW →
-1550 CTCCTCATAA CCATCGCTCC ACTCTTTGCC TTCACCGTTT TCGGTTCGGT
-1500 TCTCTACATC GCAACCCGGC CCAAACCGGT TTACCTCGTT GAGTACTCAT
-1450 GCTACCTTCC ACCAACGCAT TGTAGATCAA GTATCTCCAA GGTCATGGAT
-1400 ATCTTTTATC AAGTAAGAAA AGCTGATCCT TCTCGGAACG GCACGTGCGA
-1350 TGACTCGTCG TGGCTTGACT TCTTGAGGAA GATTCAAGAA CGTTCAGGTC
-1300 TAGGCGATGA AACTCACGGG CCCGAGGGGC TGCTTCAGGT CCCTCCCCGG
-1250 AAGACTTTTG CGGCGGCGCG TGAAGAGACG GAGCAAGTTA TCATTGGTGC
-1200 GCTAGAAAAT CTATTCAAGA ACACCAACGT TAACCCTAAA GATATAGGTA
-1150 TACTTGTGGT GAACTCAAGC ATGTTTAATC CAACTCCATC GCTCTCCGCG
-1100 ATGGTCGTTA ACACTTTCAA GCTCCGAAGC AACGTAAGAA GCTTTAACCT
-1050 TGGTGGCATG GGTTGTAGTG CCGGCGTTAT AGCCATTGAT CTAGCAAAGG
-1000 ACTTGTTGCA TGTCCATAAA AATACGTATG CTCTTGTGGT GAGCACAGAG
 -950 AACATCACTT ATAACATTTA CGCTGGTGAT AATAGGTCCA TGATGGTTTC
 -900 AAATTGCTTG TTCCGTGTTG GTGGGGCCGC TATTTTGCTC TCCAACAAGC
 -850 CTGGAGATCG TAGACGGTCC AAGTACGAGC TAGTTCACAC GGTTCGAACG
 -800 CATACCGGAG CTGACGACAA GTCTTTTCGT TGCGTGCAAC AAGGAGACGA
 -750 TGAGAACGGC AAAATCGGAG TGAGTTTGTC CAAGGACATA ACCGATGTTG
 -700 CTGGTCGAAC GGTTAAGAAA AACATAGCAA CGTTGGGTCC GTTGATTCTT
 -650 CCGTTAAGCG AGAAACTTCT TTTTTTCGTT ACCTTCATGG GCAAGAAACT
 -600 TTTCAAAGAT AAAATCAAAC ATTACTACGT CCCGGATTTC AAACTTGCTA
 -550 TTGACCATTT TTGTATACAT GCCGGAGGCA GAGCCGTGAT TGATGTGCTA
 -500 GAGAAGAACC TAGCCCTAGC ACCGATCGAT GTAGAGGCAT CAAGATCAAC
```

Figure 2 Continued ***Brassica napus FAE1*** **promoter:**

```
-450 GTTACATAGA TTTGGAAACA CTTCATCTAG CTCAATATGG TATGAGTTGG
-400 CATACATAGA AGCAAAAGGA AGGATGAAGA AAGGTAATAA AGTTTGGCAG
-350 ATTGCTTTAG GGTCAGGCTT TAAGTGTAAC AGTGCAGTTT GGGTGGCTCT
-300 AAACAATGTC AAAGCTTCGA CAAATAGTCC TTGGGAACAC TGCATCGACA
-250 GATACCCGGT CAAAATTGAT TCTGATTCAG GTAAGTCAGA GACTCGTGTC
-200 CAAAACGGTC GGTCCTAATA AACGATGTTT GCTCTCTTTC GTTTCTTTTT
-150 ATTTGTTATA ATAATTTGAT GGCTACGATG TTTCTCTTGT TTGTTATGAA
-100 TAAAGAATGC AATGGTGTTC TAGTATTTGA TTGTTTTACA TGTATGTATC
 -50 TCTTATTTAC ATGAAATTTT TAAACGCCTA AAAAAAAAAA CGGAATTCCG
                                  ← BnproRV
   1 ATGACGTCCA TTAACGTAAA GCTCCTTTAC CATTACGTCA TAACCAACCT
  51 TTTCAACCTT TGCTTCTTTC CGTTAACGGC GATCGTCGCC GGAAAAGCCT
     ← Bnwalk2
 101 ATCGGCTTAC CATAGACGAT CTTCACCACT TATACTATTC CTATCTCCAA
 151 CACAACCTCA TAACCATCGC TCCACTCTTT GCCTTCACCG
       ← Bnwalk1
```

Figure 3. ***Lunaria annua FAE1*** **promoter:**
(Length: 1069 bp)

```
-1100                          CG CCGGGGAGTT TCAGCTTAAC CGGTAAAATT
                                                    LaproFW →
-1050 GGCCTGTACA TATATTTACC ACTGAGTAAA GACATCAGTT AATGATTTGT
-1000 TGTTACTCAA TTGGGCTAAG TGTATTATTA TATGTGTTGT ATATAATAAA
 -950 GGTAGAACGT AAATTTACTA AGAATGTGTT TTTCCAATGT GATTGCTCTT
 -900 TGGCCTCTTA GGTTTGAATC CTACTCGAGA AGACTAATTT TAATTTACTG
 -850 GCAAAAATAG AAATCAATTT ATAAGTGTTT AAACAAATCG ATGGTATAAC
 -800 TGATTAGTGA TCACTCTTAG GTTTTGATCC AACTCGAGTA TTGAGTATTG
 -750 AACGCTTTTT TTAAATAAAA TCTTGATTTT TAAATTGGTT TTTTGAGTAA
 -700 AAAAGTTCTT AATATTTTCT CTTTGTTTTA ATGGGTTTGT TTTGCATTTT
 -650 ATAAGCTTAA TTTTTCTAAT TTAATATTTT ATCTATCATC GTCCGTAAAG
 -600 TTTTATTTGG CACAAACTTG TTTTACTTTT CTACCTTATA ATTTGGGAAC
 -550 TGGTTGAGTC AAAGCGTACC GGACAAATAT GTTTTATATT CTTATTTAAG
 -500 AATTAACACT CATCTCATAA TTAGTCAGAG GCTAGGGAGA TTCAGCCAAT
 -450 CAATGCTAAC AACAAAATTC TCTTAATGAT CTAACGATGC TATTTAATAT
 -400 TCGGATCAGT ATTCTTAAAT AAGAATATAA AACTAATTCA ATAGTTACAG
 -350 ATAAAAACTT ATATAGACTT TTTTATTTGG AATATAAAAG TATCAATATA
 -300 TTATAGACAA TATTTATAAC GTTAAAAATA CAATATTTAT ATTTTTTATA
 -250 TATTTATTTC AAATTGAAAA GCATTACTTC TATCGAAATG AATTTTAGTA
 -200 TATTAATTAA TATTTTTTTA ATCGGACTAC TTTCCTATTT TGGCACCTTT
 -150 CATCTGACTA CTAATTTATT TCAATGTGTA TGCATGCATG AGCATGAGTA
```

Figure 3 Continued. ***Lunaria annua FAE1*** **promoter:**

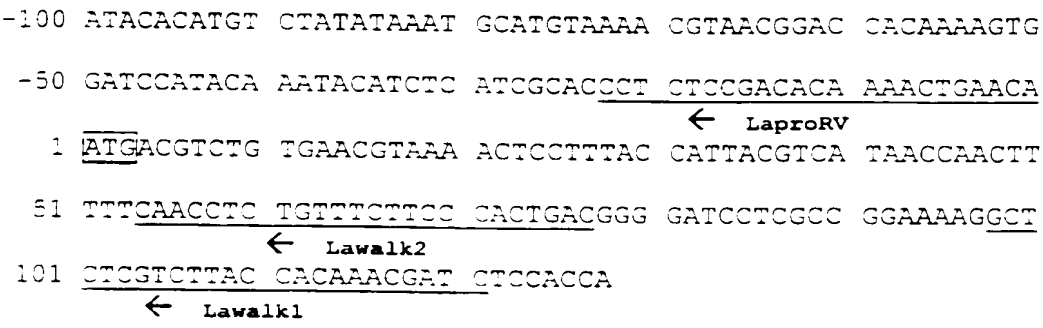


Figure 4: Alignment of *A.t.*, *L.a.* and *B.n. FAE1* promoters

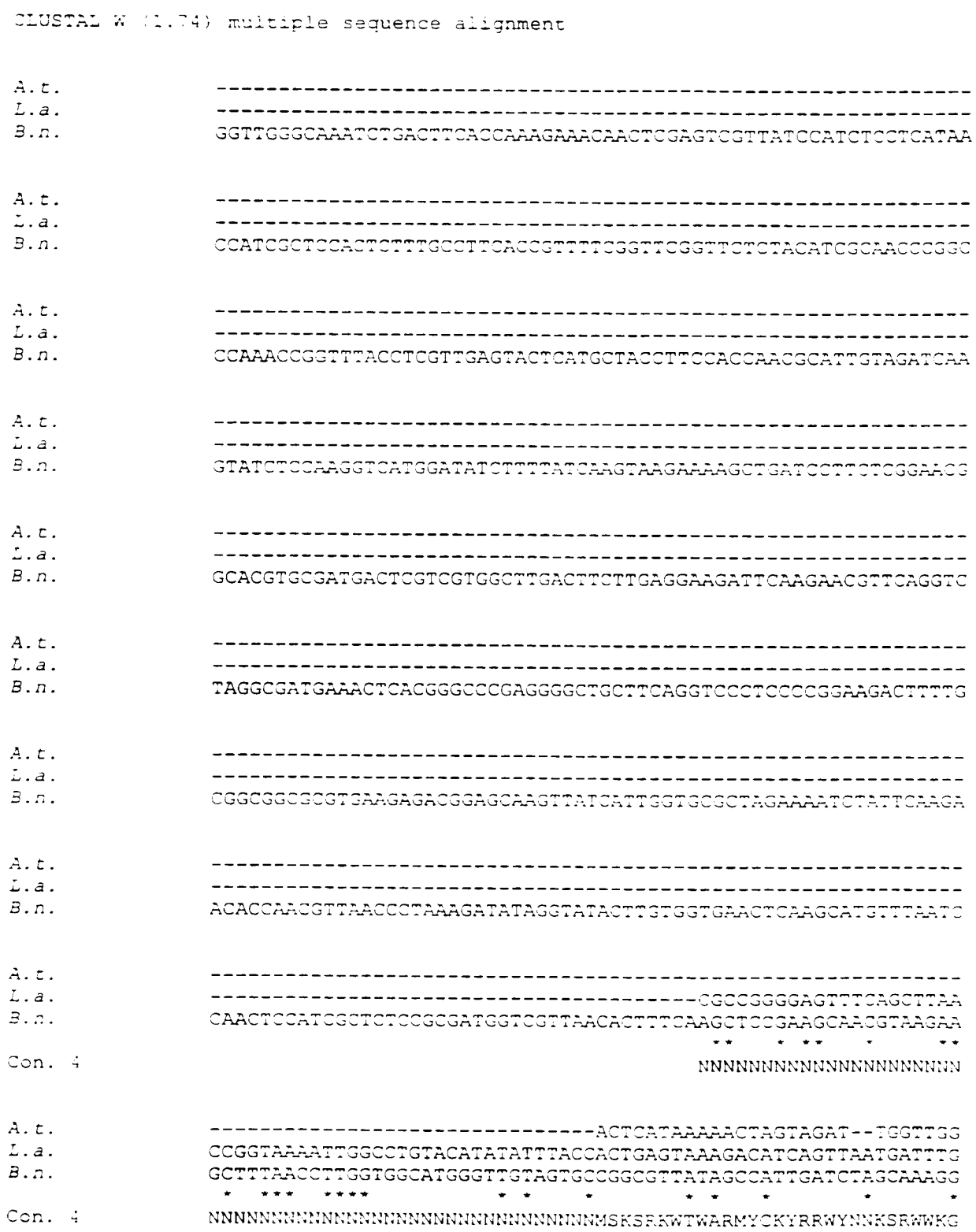


Figure 4 Continued: Alignment of *A.t.*, *L.a.* and *B.n. FAE1* promoters

```
A.t.        TTGGTTTCCA--TGTACCAGAAGGCTTACCCTAT-TAGTTGAAAGTTGAAACTTTGTTCC
L.a.        TTGTTACTCAATTGGGCTAAGTGTATTATTATAT-GTGTTGTATATAATAAAGGTAGAAC
B.n.        ACTTGTTGCATGTCCATAAAAATACGTATGCTCTTGTGGTGAGCACAGAGAACATCACTT
                      **  *       *         **   * *    * **           *    *

Con.4       WYKKKWYBCANNTSBRYHARRWKDMKTAYBMTMTNKWGKTGWRHRYWRWRAMBDTVDHHY

A.t.         CTACTCAATTCCTAGTTGTGTAAATGT---ATGTATATGTAAT---GCGTATAAAACGTA
L.a.         GTAA--ATTTACTAAGAATGTGTTTTTCCAATGTGATTGCTCTTTGGCCTCTTAGGTTTG
B.n.         ATAA-CATTTACGCTGGTGATAATAGGTCCATGATGGTTTCAAATTGCTTGTTCCGTGTT
              **  ** **  *          *         * ***     *       * ** * *        *

Con.4        VTAMNNAWTTMCMMDKDDKRTRWWWKKNNNATGWDDDTKYHMWNNNGCBTVTWMVRYKTD

A.t.         GTACTTAAATGACTAGGAGTGGTTCTTGAGACCGATGAGAGATGGGAG-CAGAACTAAAG
L.a.         AATCCTACTCGAGAAG-ACTAATTTTAATTTACTGGCAAAAATAGAAA-TCAATTTATAA
B.n.         GGTGGGGC-CGCTATTTTGCTCTCCAACAAGCCTGGAGATCGTAGACGGTCCAAGTACGA
                      *                 *                 *       * *       *  **

Con.4        RDWSBKRMNYGMBWWKNWSYDVTYYWWVWDDMCKRKVRRWVRTRGRMRNYMVAWBTAHRR

A.t.          AT--GATGACATAATTA------AGAACGAATTTGA-AAGG-CTCTTAGGTTTGAATCCT
L.a.          GT--GTTTAAACAAATCGATGGTATAACTGATTAGT-GATCACTCTTAGGTTTTGATCCA
B.n.          GCTAGTTCACACGGTTCGAACGCATACCGGAGCTGACGACAAGTCTTTTCGTTGCGTGCA
                  * * * *    * **  * * * * *   *   *   *  * ****    **   * *

Con.4         RYNNGWTBAMAYRRWTMNNNNNNNAKAMCKRAKYWGWNRABVNSTCTTWKSKTTKVRTSCW

A.t.          ATTCGAGAATGTTTTTGTCAAAGATAGTGGCGATTTTGAACCAAAGAAAACATTTAAA-A
L.a.          ACTCGAGTATTGAGTATTGAACGCTT-------TTTTTAAATAAAATCTTGATTTTTA-A
B.n.          A--CAAGGA-GACGATGAGAACGGCAA-----AATCGGAGTGAGTTTGTCCAAGGACATA
              * ** ** **          ** *         * *   *   *         *    *-*

Con.4         ANNCRAGDANKDHKWWKWSAAMGVYWNNNNNNNNWTYKKARHBARWDWVWHSAWKKWHANA

A.t.          AATCAGTATCCGGTTAC----GTTCATGCAAATAGAAAGTGGTCTA---GGATCTGATT-
L.a.          ATTGGTTTTTTGAGTAAAAAAGTTCTTAATATTTTCTCTTTGTTTTAATGGGTTTGTTT-
B.n.          ACCGATGTTGCTGGTCGAACGGTTAAGAAAAACATAGCAACGTT-----GGGTCCGTTGA
              *       *     *   **  ***       *           **  *     **  *  *  *

Con.4         AHYSRKKWTBYKRKTMVNNNNGTTMWKRMWAWYWKMDMDWBGTYNNNNNGGRTYYGWTKN


A.t.           GTAATTTTAGA--GTTAAAGAGTCTC--TTAAGATTCAATCCTGGCT-GTGTACAAAACT
L.a.           TGCATTTTATAAGCTTAATTTTTCTAATTTAATATTTTATCTATCATCGTCCGTAAAGTT
B.n.           TTCTTCCGTTAAGCGAGAAACTTCTT--TTTTTCGTTA--CCTTCATGGGCAAGAAACTT
                   *     ****    *     ***    **      *   ***      *  *      ***   *

Con.4          KKMWTYYKWKANNCKWRAWDHKTCTHNNTTWWKMKTYWNNCYWKSMTNGKSHRBAAAVYT

A.t.           ACAAATAATATA----TTTTAGACTATTTGGCCTTAACTAAACTTCCA-CTCATTATTTA
L.a.           TTATTTGGCACAAACTTGTTTTACTTTTCTACCTTA--TAATTTGGGAACTGGTTGAGT-
B.n.           TTCAAAGATAAAATCAAACATTACTACGTCCCGGATTTCAAACTTGCTATTGACCATTTT
                   * **  *       ***       *         **   *     *  *        *

Con.4          WYMWWWRRYAHANNNNWDYWWKACTWYKYBVCSKWWNNYAAWYTKSSWNYTSRYYRWKTN

A.t.           -CTGAGGTTAGAGAA--TAGACTTGCGAATAAACACATTCCCGAGAAATACTCATGATCC
L.a.           -CAAAGCGTACCGGA--CAAATATGTTT-TATATTCTTATTTAAGAATTAACACTCATCT
B.n.           TGTATACATGCCGGAGGCAGAGCCGTGATTGATGTGCTAGAGAAGAACCTAGCCCTAGCA
                     *    * *    * *    *     *       *      ****          * *

Con.4          NSWRWRSDTRSMGRANNYARABHYGYKWNTRWWBWSHTWBHBRAGAAHYWMBMMYBAKCH
```

Figure 4 Continued: Alignment of *A.t.*, *L.a.* and *B.n. FAE1* promoters

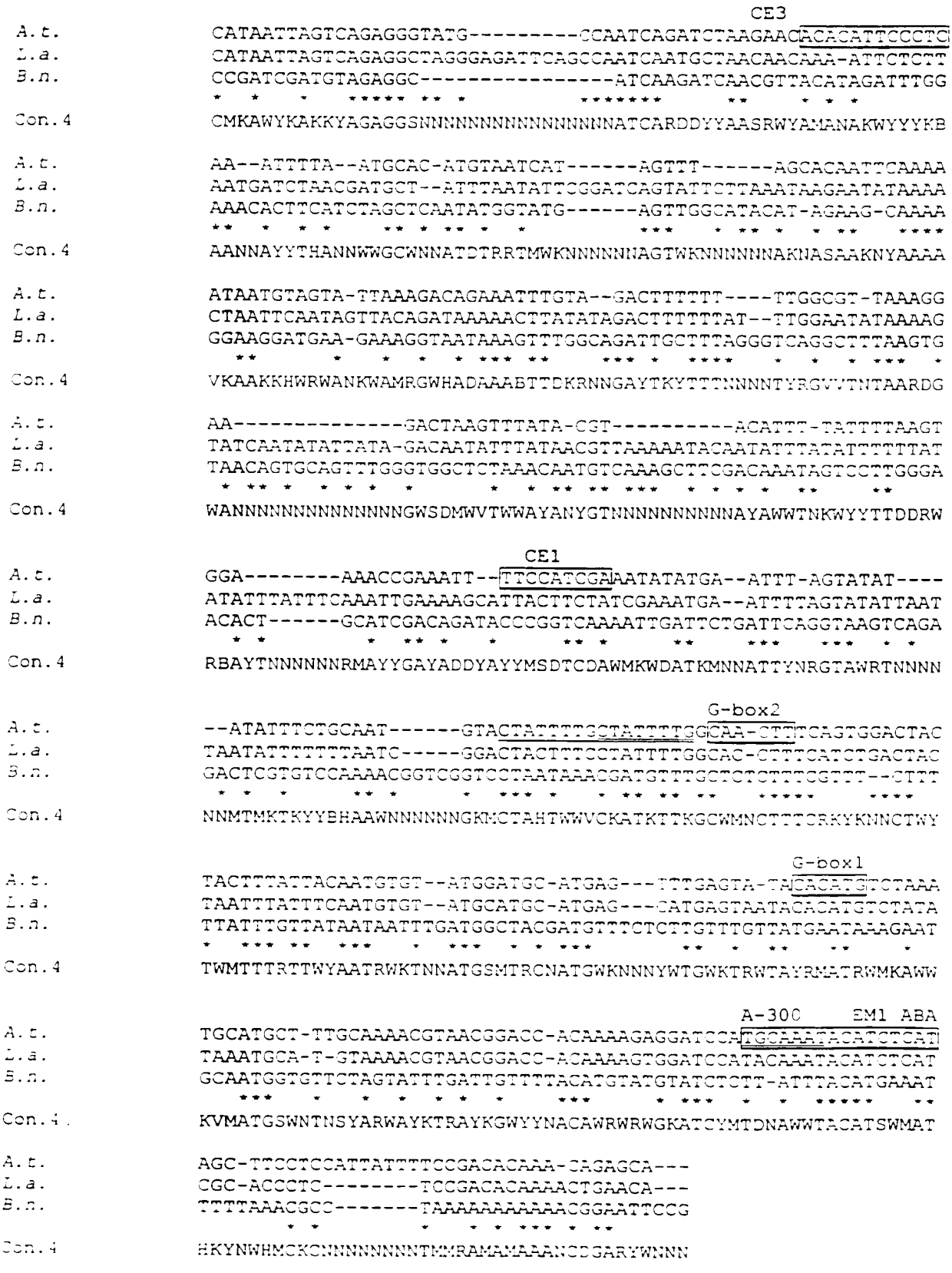


Figure 5: Alignment of *A.t.* and *L.a. FAE1* promoters

CLUSTAL W (1.74) multiple sequence alignment

```
A.t.                ----------------------------------------------------ACTCATAA
L.a.                CGCCGGGGAGTTTCAGCTTAACCGGTAAAATTGGCCTGTACATATATTTACCACTGAGTA
                                                                        *** * *
Con.5                                                                   ACTSAKWA

A.t.                AAACTAGTAGAT--TGGTTGGTTGGTTTCCA--TGTACCAGAAGGCTTACCCTATTAGTT
L.a.                AAGACATCAGTTAATGATTTGTTGTTACTCAATTGGGCTAAGTGTATTATTATATGTGTT
                    **   *  ** *  ** ** **** *   **  **  * *   *  ***   *** ***
Con.5               AARMYAKYAGWTNNTGRTTKGTTGKTWYYCANNTGKRCYARRWGKMTTAYYMTATKWGTT

A.t.                GAAAGTTGAAACTTTGTTCCCTACTCAATTCCTAGTTGTGTAAATGT---ATGTATATGT
L.a.                GTATATAATAAAGGTAGAACGTAA--ATTTACTAAGAATGTGTTTTTCCAATGTGATTGC
                    * *  *   **   *   * **   * ** ***    ***   * *   ****   **
Con.5               GWAWRTWRWAAMKKTRKWMCSTAMNNAWTTMCTARKWRTGTRWWTKTNNNATGTRWWTGY

A.t.                AAT---GCGTATAAAACGTAGTACTTAAATGACTAGGAGTGGTTCTTGAGACCGATGAGA
L.a.                TCTTTGGCCTCTTAGGTTTGAATCCTACTCGAGAAG-ACTAATTTTAATTTACTGGCAAA
                      *   ** * * *    *   * **   **  ** * *  ** *     *    * *
Con.5               WMTNNNGCSTMTWARRYKTRRWWCYTAMWYGASWAGNASTRRTTYTWRWKWMCKRKSARA

A.t.                GATGGGAGCAGAACTAAAGATGATGACATAATTA------AGAACGAATTTGAAAGG-CT
L.a.                AATAGAAATCAATTTATAAGTGTTTAAACAAATCGATGGTATAACTGATTAGTGATCACT
                     ** * *    *  ** *  ** * * * ** *       * ***  *** * *   **
Con.5               RATRGRARYMRAWYTAWARRTGWTKAMAYAAWTMNNNNNNNAKAACKRATTWGWRAKSNCT

A.t.                CTTAGGTTTGAATCCTATTCGAGAATGTTTTTGTCAAAGATAGTGGCGATTTTGAACCAA
L.a.                CTTAGGTTTTGATCCAACTCGAGTATTGAGTATTGAACGCTT-------TTTTTAAATAA
                    *********  **** * ***** **    *  * ** * *         **** **  **
Con.5               CTTAGGTTTKRATCCWAYTCGAGWATKKWKTWKTSAAMGMTWNNNNNNNTTTTKAAMYAA

A.t.                AGAAAACATTTAAAAAATCAGTATCCGGTTAC----GTTCATGCAAATAGAAAGTGGTCT
L.a.                AATCTTGATTTTTAAATTGGTTTTTTGAGTAAAAAAGTTCTTAATATTTTCTCTTTGTTT
                    *     ****  *** *   * *  *  **     **** *   * *      * ** *
Con.5               ARWMWWSATTTWWAAAWTSRKTWTYYGRKTAMNNNNGTTCWTRMWAWTWKMWMKTKGTTT

A.t.                A---GGATCTGATTGTAATTTTAGA--CTTAAAGAGTCTC--TTAAGATTCAATCCTGGC
L.a.                TAATGGGTTTGTTTTGCATTTTATAAGCTTAATTTTTCTAATTTAATATTTTATCTATCA
                        ** * ** **   ****** *  *****    ***   **** ***  ***
Con.5               WNNNGGRTYTGWTTKKMATTTTAKANNCTTAAWKWKTCTMNNTTAAKATTYWATCYWKSM

A.t.                T-GTGTACAAAACTACAAATAATATA----TTTTAGACTATTTGGCCTTAACTAAACTTC
L.a.                TCGTCCGTAAAGTTTTATTTGGCACAAACTTGTTTTACTTTTCTACCTTA--TAATTTGG
                    * **    ***  *  *  *   * *    * **  *** **   *****  ***  *
Con.5               TNGTSYRYAAARYTWYAWWTRRYAYANNNNTKTTWKACTWTTYKRCCTTANNTAAWYTKS

A.t.                CA-CTCATTATTTACTGAGGTTAGAGAATAGACTTGCGAATAAACACATTCCCGAGAAAT
L.a.                GAACTGGTTGAGT-CAAAGCGTACCGGACAAATATGTTT-TATATTCTTATTTAAGAATT
                     * **  **   * *  **  **  * * * *  **    ** *  * *     **** *
Con.5               SANCTSRTTRWKTNCWRAGSKTASMGRAYARAYWTGYKWNTAWAYWCWTWYYYRAGAAWT

                                                          -400
A.t.    -432        ACTCATGATCCCATAATTAGTCAGAGGGTATG---------CCAATCAGATCTAAGAACA
L.a.                AACACTCATCTCATAATTAGTCAGAGGCTAGGGAGATTCAGCCAATCAATGCTAACAACA
                    *    * *** ********************* ** *          *******   **** ****
Con.5               AMYMMTSATCYCATAATTAGTCAGAGGSTAKGNNNNNNNNNCCAATCARWKCTAASAACA
```

Figure 5 Continued: Alignment of *A.t.* and *L.a. FAE1* promoters

```
A.t.    -381    CACATTCCCTCAA--ATTTTA--ATGCACATGTAATCAT------AGTTT------AGCA
L.a.            AA-ATTCTCTTAATGATCTAACGATGCT-ATTTAATATTCGGATCAGTATTCTTAAATAA
                 * **** ** **  ** * *  **** ** **** *       *** *      * *
Con.5           MANATTCYCTYAANNATYTWANNATGCWNATKTAATMWTNNNNNNAGTWTNNNNNNAKMA

A.t.    -337    CAATTCAAAAATAATGTAGTA-TTAAAGACAGAAATTTGTA--GACTTTTTT--TTGGCG
L.a.            GAATATAAAACTAATTCAATAGTTACAGATAAAAACTTATATAGACTTTTTTATTTGGAA
                 ***  **** **** * ** *** *** * *** ** **  ********* ****
Con.5           SAATWYAAAAMTAATKYARTANTTAMAGAYARAAAYTTRTANNGACTTTTTTNNTTGGMR

A.t.    -282    T-TAAAGGAA-------------GACTAAGTTTATA-CGT----------ACATTT-TAT
L.a.            TATAAAAGTATCAATATATTATAGACAATATTTATAACGTTAAAAATACAATATTTATAT
                * **** * *              *** *  ****** ***          * **** ***
Con.5           TNTAAARGWANNNNNNNNNNNNNGACWAWRTTTATANCGTNNNNNNNNNNAYATTTNTAT

A.t.    -247    TTTAAGTGGA--------AAACCGAAATT--TTCCATCGAAATATATGAATTT-AGTATA
L.a.            TTTTTATATATTTATTTCAAATTGAAAAGCATTACTTCTATCGAAATGAATTTTAGTATA
                ***   *  *         ***  ****    ** * ** *   * ******** ******
Con.5           TTTWWRTRKANNNNNNNNAAAYYGAAAWKNNTTMCWTCKAWMKAWATGAATTTNAGTATA

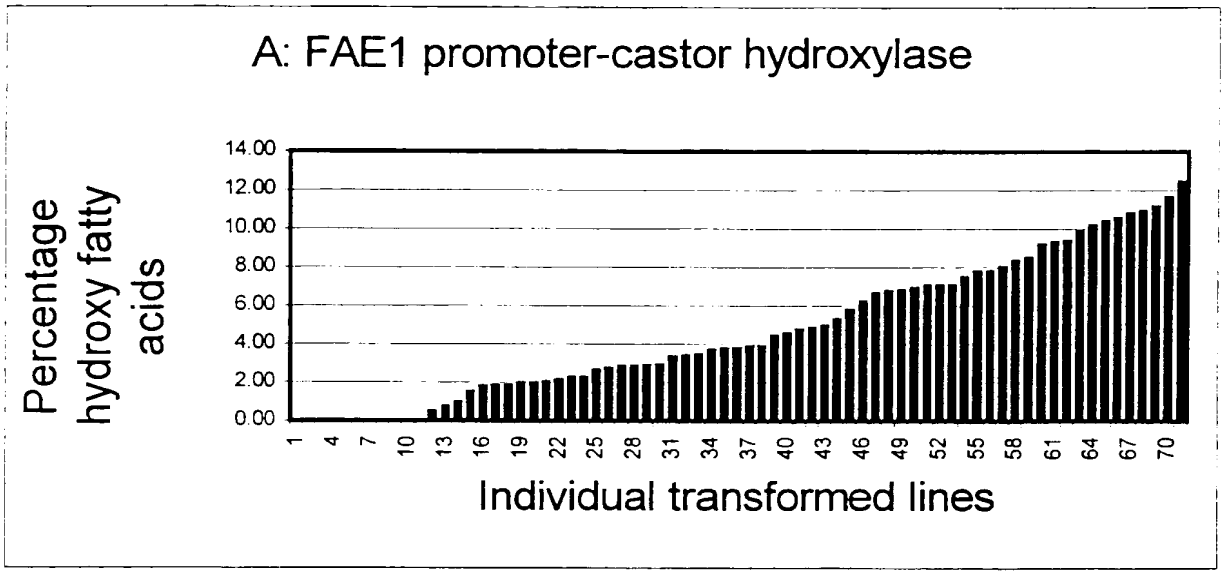
A.t.    -198    T------ATATTTCTGCAAT-GTACTATTTTGCTATTTTGGCAACTTTCAGTGGACTACT
L.a.            TTAATTAATATTTTTTTAATCGGACTACTTTCCTATTTTGGCACCTTTCATCTGACTACT
                *      ****** *  *** * **** *** ********** ******   *******
Con.5           TNNNNNNATATTTYTKYAATNGKACTAYTTTSCTATTTTGGCAMCTTTCAKYKGACTACT

A.t.    -145    ACTTTATTACAATGTGTATGGATGCATGAGTTTGAGTA-TACACATGTCTAAATGCATGC
L.a.            AATTTATTTCAATGTGTATGCATGCATGAGCATGAGTAATACACATGTCTATATAAATGC
                * ****** ********** ********* ****** ********** **  ****
Con.5           AMTTTATTWCAATGTGTATGSATGCATGAGYWTGAGTANTACACATGTCTAWATRMATGC

A.t.    -86     TTTGCAAAACGTAACGGACCACAAAAGAGGATCCATGCAAATACATCTCATAGCTTCCTC
L.a.            AT-GTAAAACGTAACGGACCACAAAAGTGGATCCATACAAATACATCTCATCGCACCCTC
                 * * ********************** ******** ************** **  ****
Con.5           WTNGYAAAACGTAACGGACCACAAAAGWGGATCCATRCAAATACATCTCATMGCWYCCTC

A.t.    -26     CATTATTTTCCGACACAAA-CAGAGCA
L.a.            --------TCCGACACAAAACTGAACA
                        *********** * ** **
Con.5           NNNNNNNNTCCGACACAAANCWGARCA
```

Figure 6


A: FAE1 promoter-castor hydroxylase
Percentage hydroxy fatty acids
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43 46 49 52 55 58 61 64 67 70
Individual transformed lines

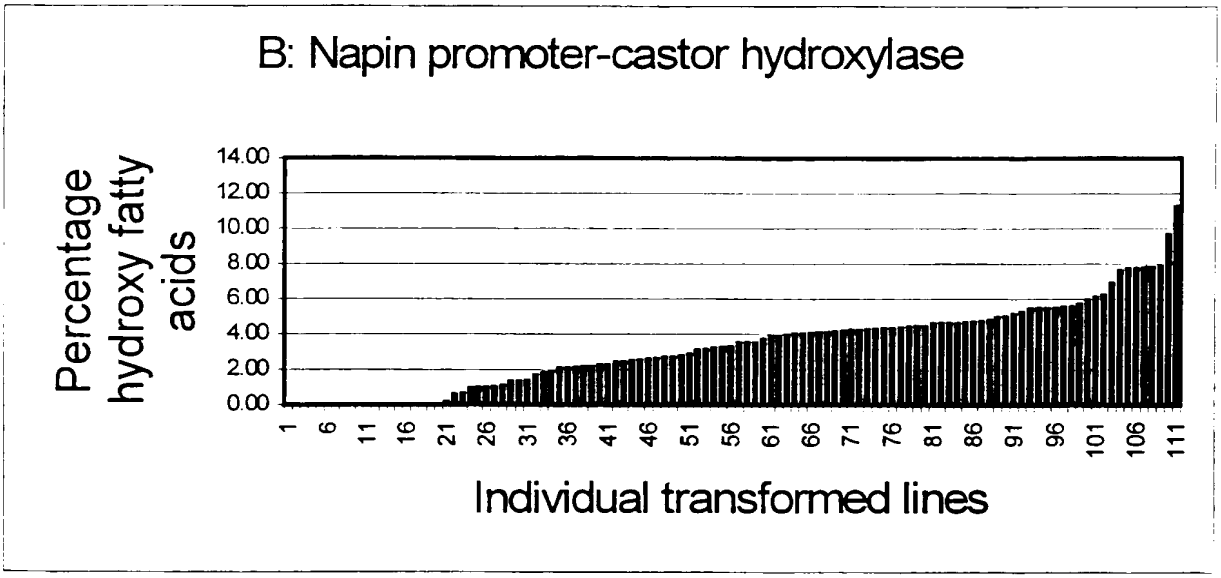

B: Napin promoter-castor hydroxylase
Percentage hydroxy fatty acids
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
1 6 11 16 21 26 31 36 41 46 51 56 61 66 71 76 81 86 91 96 101 106 111
Individual transformed lines

Figure 7: Alignment of *B.n.* and *L.a. FAE1* promoters

```
CLUSTAL W (1.81) multiple sequence alignment

BnFAE1          GGTTGGGCAAATCTGACTTCACCAAAGAAACAACTCGAGTCGTTATCCATCTCCTCATAA 60
LaFAE1          ------------------------------------------------------------

BnFAE1          CCATCGCTCCACTCTTTGCCTTCACCGTTTTCGGTTCGGTTCTCTACATCGCAACCCGGC 120
LaFAE1          ------------------------------------------------------------

BnFAE1          CCAAACCGGTTTACCTCGTTGAGTACTCATGCTACCTTCCACCAACGCATTGTAGATCAA 180
LaFAE1          -------------CGCCGGGGAGT-TTCAGCTTAACCGGTAAAATTGGCCTGTACATATA 46

BnFAE1          GTATCTCCAAGGTCATGGATATCTTTTATCAAGTAAGAAAAGCTGATCCTTCTCGGAACG 240
LaFAE1          TTTACCACTGAGT-AAAGACATCAGTTAATGATTT------GTTGTTACTCAATTGGGCT 99

BnFAE1          GCACGTGCGATGACTCGTCGTGGCTTGACTTCTTGAGGAAGATTCAAGAACGTTCAGGTC 300
LaFAE1          AAGTGTATTATTATATGTGTTG-------TATATAATAAAGGT---AGAACGT--AAATT 147

BnFAE1          TAGGCGATGAAACTCACGGGCCCGAGGGGCTGCTTCAGGTCCCTCCCCGGAAGACTTTTG 360
LaFAE1          TA--CTAAGAATGTGTTTTTCCAATGTGATTGCTCTTTGGCCTCTTAGGTTTGAATCCTA 205

BnFAE1          CGGCGGCGCGTGAAGAGACGGAGCAAGTTATCATTGGTGCGCTAGAAAATCTATTCAAGA 420
LaFAE1          CT--------CGAGAAGACTAATTTTAAT-TTACTGGCAAAAATAGAAATCAATTTATAA 256

BnFAE1          ACACCAACGTTAACCCTAAAGATATAGGTATACTTGTGGTGAACTCAAGCATGTTTAATC 480
LaFAE1          GTGTTTAAACAAATC--GATGGTATAACTG-ATTAGTGATCACTCTTAGGTT--TTGATC 311

BnFAE1          CAACTCCATCGCTCTCCGCGATGGTCGTTAACACTTTCAAGCTCCGAAGCAACGTAAGAA 540
LaFAE1          CAACTCGAGTATTG---------AGTATTGAACGCTTT-----TTTTAAATAAAATCTTGA 358

BnFAE1          GCTTTAACCTTGGTGGCATGGGTTGTAGTGCCGGCGTTATAGCCATTGATCTAGCAAAGG 600
LaFAE1          TTTTTAAA-TTGGTTTTTTGAGTAAAAAAGTTCTTAATATTTTCTCTT-TGTTTTAATGG 416

BnFAE1          ACTTGTTGCATGTCC-ATAAAAATACGTATGCTCTTGTGGTGAGCACAGAGAACATCACT 659
LaFAE1          GTTTGTTTTGCATTTTATAAGCTTAATTTTTCTAATTTAAT-ATTTTATCTATCATCGTC 475

BnFAE1          TATAACATTTACGCTGGTGATAATAGGTCCATGATGGTTTCAAATTGCTTGTTCCGTGTT 719
LaFAE1          CGTAAAGTTT----------TATTTGGCACAAACTTGTTTTA---CTTTTCTACCTTATA 522

BnFAE1          GGTGGGGCCGCTATTTTGCTCTCCAACAAGCCTGGAGATCGTAGACGGTCCAAGTACGAG 779
LaFAE1          ATTTGGGA-ACTGGTTGAGTCA-----AAGCGTACCGGACAAATATGTTTTATATTC--- 573

BnFAE1          CTAGTTCACACGGTTCGAACGCATACCGGAGCTGACGACAAGTCTTTTCGTTGCGTGCAA 839
LaFAE1          -TTATTTA-AGAATTAACACTCATCTCATAATTAGTCAGAGGC-------TAGGGAGATT 624

BnFAE1          CAAGGAGACGATGAGAACGGCAAAATCGGAGTGAGTTTGTCCAAGGACATAACCGATGTT 899
LaFAE1          CAGCCAATCAATGCTAACAACAAAATTCTCTTAA--TGATCTAACGATGCTATTTAATAT 682
```

Figure 7 Continued: Alignment of *B.n.* and *L.a. FAE1* promoters

```
BnFAE1          GCTGGTCGAACGGTTAAGAAAAACATAGCAACGTTGGGTCCGTTGATTCTTCCGTTA-AG 958
LaFAE1          TCGGATCAGTATTCTTAAATAAGAATATAAA----------ACTAATTCAATAGTTACAG 732
                 * * **       * * * **  ***  **           * ****    **** **

BnFAE1          CGAGAAACTTCTTTTTTTCGTTACCTTCATGGGCAAGAAACTTTTCAAAGATAAAATCAA 1018
LaFAE1          ATAAAAACTTATATAGACTTTTTTATTTG-GAATATAAAAGTATCAATATATTATAGACA 791
                  * ****** * *      **   **   *   *  *** * *  * * ** * *   *

BnFAE1          ACATTACTACGTCCCGGATTTCAAACTTGCTATTGACCATTTTTGTATACATGCCGGAGG 1078
LaFAE1          ATATTTATA------ACGTTAAAAATACAATATTTATATTTTTTATATATTTATTTCAAA 845
                * ***  **          **  ***     **** *   ***** **** *     *

BnFAE1          CAGAGCCGTGATTGATGTGCTAGAGAAGAACCTAGCCCTAGCACCGATCGATGTAGAGGC 1138
LaFAE1          TTGAAAAGCATTACTTCTATCGAAATGAATTTTAGT----ATATTAATTAATATTTTTTT 901
                  **   *   *   * *     *    *   ***       *   **  ** *

BnFAE1          ATCAAGATCAACGTTACATAGATTTGGAAACACTTCATCTAGCTCAATATGGTATGAGTT 1198
LaFAE1          AATCGGACTACTTTCCTAT----TTTGGCACCTTTCATCTGACT-------------ACT 944
                *    **  *   *   **    ** *  **  *******  **              *

BnFAE1          GGCATACATAGAAGCAAAAGGAAGGATGAAGAAAGGTAATAAAGTTTGGCAGATTGCTTT 1258
LaFAE1          AATTTATTTCAATGTGTATGCATGCATGAGCATGAGTAATA--------CACATGTCTAT 996
                    **  *  * *   * * * * ****  *   ******         ** **  ** *

BnFAE1          AGGGTCAGGCTTTAAGTGTAACAGTGCAGTTTGGGTGGCTCTAAACAATGTCAAAGCTTC 1318
LaFAE1          ATAAATGCATGTAAAACGTAACGG-ACCACAAAAGTGGATCCATACAAATACATCTCATC 1055
                *          * **  ***** *  *       **** ** * ****   **   * **

BnFAE1          GACAAATAGTCCTTGGGAACACTGCATCGACAGATACCCGGTCAAAATTGATTCTGATTC 1378
LaFAE1          G-CACCCTCTCCGACACAAAACTGAACA------------------------------- 1082
                * **     ***     ** **** *

BnFAE1          AGGTAAGTCAGAGACTCGTGTCCAAAACGGTCGGTCCTAATAAACGATGTTTGCTCTCTT 1438
LaFAE1          ------------------------------------------------------------

BnFAE1          TCGTTTCTTTTTATTTGTTATAATAATTTGATGGCTACGATGTTTCTCTTGTTTGTTATG 1498
LaFAE1          ------------------------------------------------------------

BnFAE1          AATAAAGAATGCAATGGTGTTCTAGTATTTGATTGTTTTACATGTATGTATCTCTTATTT 1558
LaFAE1          ------------------------------------------------------------

BnFAE1          ACATGAAATTTTTAAACGCCTAAAAAAAAAAAACGGAATTCCG 1600
LaFAE1          -------------------------------------------
```

Figure 8: Alignment of *B.n.* and *A.t. FAE1* promoters

```
CLUSTAL W (1.81) multiple sequence alignment

AtFAE1          ------------------------------------------------------------
BnFAE1          GGTTGGGCAAATCTGACTTCACCAAAGAAACAACTCGAGTCGTTATCCATCTCCTCATAA 60

AtFAE1          ------------------------------------------------------------
BnFAE1          CCATCGCTCCACTCTTTGCCTTCACCGTTTTCGGTTCGGTTCTCTACATCGCAACCCGGC 120

AtFAE1          ------------------------------------------------------------
BnFAE1          CCAAACCGGTTTACCTCGTTGAGTACTCATGCTACCTTCCACCAACGCATTGTAGATCAA 180

AtFAE1          ----------------------------------------------------ACTCATAAAA 10
BnFAE1          GTATCTCCAAGGTCATGGATATCTTTTATCAAGTAAGAAAAGCTGATCCTTCTCGGAACG 240
                                                                    ***  **

AtFAE1          ACTAGTAGATTGGTTGGT--TGGTTTCCATGTACCAGAAGGCTT-----ACCCTATTAGT 63
BnFAE1          GCACGTGCGATGACTCGTCGTGGCTTGACTTCTTGAGGAAGATTCAAGAACGTTCAGGTC 300
                 *  **    **  * **  *** **   *     ** * * **     **  *

AtFAE1          TGAAAGTTGAAACTT-TGTTCCCTACT--CAATTCCTAGTTGTGTAAATGTATGTATATG 120
BnFAE1          TAGGCGATGAAACTCACGGGCCCGAGGGGCTGCTTCAGGTCCCTCCCCGGAAGACTTTTG 360
                *    * *******    *  *** *    *   * *  **          * *    * **

AtFAE1          TAATG-CGTATAAAACGTAGTACTTAAATGACTAGGAGTGGTTCTTGAGACCGATGAGAG 179
BnFAE1          CGGCGGCGCGTGAAGAGACGGAGC-AAGTTATCATTGGTGCGCTAGAAAATCTATTCAAG 419
                    * **  * **  *  * *   ** * *  *   ***       * * * **   **

AtFAE1          A----TGGGAGCAGAACTAAAGATGATGACATAATTAAGAACGAATTTGAAAGGCTCTTA 235
BnFAE1          AACACCAACGTTAACCCTAAAGATATAGGTATACTTGTGG-TGAACTCAAGCATGTTTAA 478
                *           *   ********   *  *** **  *   *** *  *     * * *

AtFAE1          GGTTTGAATCCTATTCGAGAATGTTTTTGTCAAAGATAGTGGCGA-TTTTGAACCAAAGA 294
BnFAE1          ---TCCAACTCCATCGCTCTCCGCGATGGTCGTTAACACTTTCAAGCTCCGAAGCAACGT 535
                   *  **  * **         *   * ***    * * *  * *  *  *** *** *

AtFAE1          AAACATTTAAAAAATCAGTATCC--GGTTAC-GTTCATGCAA-ATAGAAAGTGGTCTAGG 350
BnFAE1          AAGAAGCTTTAACCTTGGTGGCATGGGTTGTAGTGCCGGCGTTATAGCCATTGATCTAGC 595
                **  *  *  **  *  **  *   ****   ** *  **   ****  * ** *****

AtFAE1          ATCTGATTGTAATTTTAGACTTAAAGAGTCTCTTAAGATTCAATCCTGGCTGTGTACAAA 410
BnFAE1          AAAGGACTT--GTTGCATGTCCATAAAAATACGTATGCTCTTGTGGTGAGCACAGAGAAC 653
                *   ** *    **  *     * * *    * ** * *    *  **       * **

AtFAE1          ACTACAAATAATATAT---TTTAGACTATTTGGCCTTAACTAAACTTCCACTCATTATTT 467
BnFAE1          ATCACTTATAACATTTACGCTGGTGATAATAGGTCCATGATGGTTTCAAATTGCTTGTTC 713
                *  **  **** ** *    *     ** * ** *     *   *  * *  ** **

AtFAE1          ACTGAGGTTAGAGA-ATAGACTTGCGAATAAACACATTCCCGAGAAATACTCATGATCCC 526
BnFAE1          CGTGTTGGTGGGGCCGCTATTTTGCTCTCCAACAAG--CCTGGAGATCGTAGACGGTCCA 771
                  **  * * * *        ****    ****    ** *   *      * * ***

                                                          CE3
AtFAE1          ATAATTAGTCAGAGGGTATG--CCAATCAGATCTAAGAACACACATTCCCTCAAATTTTA 584
BnFAE1          AGTACGAGCTAGTTCACACGGTTCGAACGCATACCGGAGCTGACGACAAGTCTTTTCGTT 831
                *  *  **  **     * *   * * *  **    ** *  **      **   *  *

AtFAE1          ATGCACATGTAATCATAGTTTAGCACAATTCAAAAATAATGTAGTATTAAAGACAGAAAT 644
BnFAE1          GCGTGCAACAAGGAGACGATGAGAACGGCAAAATCGGAGTGAGTTTGTCCAAGGACATAA 891
                  *  **   *      * * ** **     **    * **   *  *  *   * * *
```

Figure 8 Continued: Alignment of *B.n.* and *A.t. FAE1* promoters

```
AtFAE1          TTGTAGACTTTTTTTTGGCGTTAAAGGAAGACTAAG------TTTATACGTACATTTTAT 698
BnFAE1          CCGATGTTGCTGGTCGAACGGTTAAGAAAAACATAGCAACGTTGGGTCCGTTGATTCTTC 951
                  *  *    *  *    ** * *** ** **  **       *   * ***  *** *

AtFAE1          T-TTAAGTGGAAAACCGAAATTTTCCAT------CGAAATATATGAATTTAGTATATATA 751
BnFAE1          CGTTAAGCGAGAAACTTCTTTTTTTCGTTACCTTCATGGGCAAGAAACTTTTCAAAGATA 1011
                  ***** *  ****     **** * *      *       *  ** **   * * ***

                                                 G box 2
AtFAE1          TTTCTGCAATGTACTATTTTGCTATTTTGGCAACTTTCAGTGGACTACTACTTTAT-TAC 810
BnFAE1          AAATCAAACATTACTACGTCCCGGATTTCA-AACTTGCTATTGACCATTTTTGTATACAT 1070
                       *   *****  *  *   ***   ***** *  * *** * *  * ***  *

                                                 G-box 1
AtFAE1          AATGTGTATGGATGCATGAGTT-TGAGTATACACATGTCTAAATGCATGCTTTGCAAAAC 869
BnFAE1          GCCGGAGGCAGAGCCGTGATTGATGTGCTAGAGAAGAACCTAGCCCTAGCACCGATCGAT 1130
                   *      **  * *** *  ** *       *   *  *   *  **   *    *

AtFAE1          GTAACGG-ACCACAAAAGAGGATCCAT------GCAAATACATCTCATAGCTTCCTCCAT 922
BnFAE1          GTAGAGGCATCAAGATCAACGTTACATAGATTTGGAAACACTTCATCTAGCTCAATATGG 1190
                *** * ** *  *  * * * ***     * *** ** **  *****  *

AtFAE1          TATTTTCCGACACAAACAGA-GCA---------------------------------- 945
BnFAE1          TATGAGTTGGCATACATAGAAGCAAAAGGAAGGATGAAGAAAGGTAATAAAGTTTGGCAG 1250
                ***     * ** * * *** ***

AtFAE1          ------------------------------------------------------------
BnFAE1          ATTGCTTTAGGGTCAGGCTTTAAGTGTAACAGTGCAGTTTGGGTGGCTCTAAACAATGTC 1310

AtFAE1          ------------------------------------------------------------
BnFAE1          AAAGCTTCGACAAATAGTCCTTGGGAACACTGCATCGACAGATACCCGGTCAAAATTGAT 1370

AtFAE1          ------------------------------------------------------------
BnFAE1          TCTGATTCAGGTAAGTCAGAGACTCGTGTCCAAAACGGTCGGTCCTAATAAACGATGTTT 1430

AtFAE1          ------------------------------------------------------------
BnFAE1          GCTCTCTTTCGTTTCTTTTTATTTGTTATAATAATTTGATGGCTACGATGTTTCTCTTGT 1490

AtFAE1          ------------------------------------------------------------
BnFAE1          TTGTTATGAATAAAGAATGCAATGGTGTTCTAGTATTTGATTGTTTTACATGTATGTATC 1550

AtFAE1          ------------------------------------------------
BnFAE1          TCTTATTTACATGAAATTTTTAAACGCCTAAAAAAAAAAAACGGAATTCCG 1600
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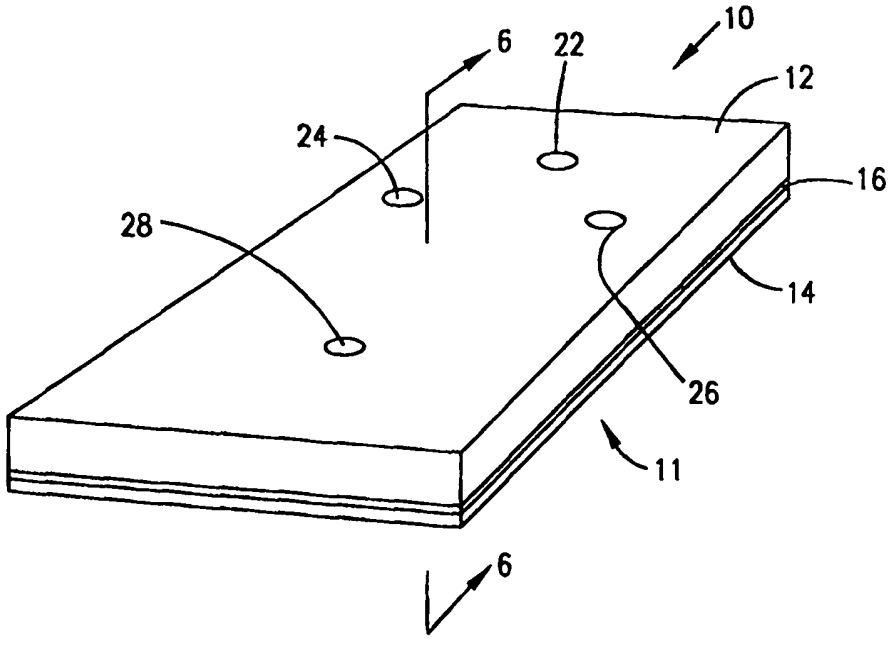
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(54) Title: METHODS FOR FABRICATING ENCLOSED MICROCHANNEL STRUCTURES

(57) Abstract

Methods are provided for the fabrication of polymeric microchannel structures having enclosed microchannels of capillary dimension. The microchannel structures are constructed of a base member (12) and a cover member (14), sealed together. A microchannel structure having walls of a plastic material is formed in a generally planar surface of at least the base member. The cover member has at least one generally planar surface, and the microchannel structure is enclosed by bonding the planar surfaces of the cover member and the base member together. In some embodiments the surfaces of the cover member and base member are both of plastic material, and are directly thermally bonded. In some embodiments a bonding material is applied to one of the surfaces prior to bringing the surfaces together. Suitable bonding material are disclosed.



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METHODS FOR FABRICATING ENCLOSED MICROCHANNEL STRUCTURES

BACKGROUND

This invention relates to construction of microchannel structures for use in microfluidic manipulations.

Microchannel structures are of great interest for applications involving the manipulation of small fluid volumes, such as chemical and biochemical analysis. Various microchannel structures having channel dimensions on the order of one or a few millimeters have been used for chemical and biochemical assays.

These structures are typically produced by injection molding using various thermoplastic polymers. Injection molding is an economical process, and a variety of thermoplastics having good optical and mechanical properties can be processed by injection molding to form the desired structures. The injection molding process involves introducing a molten thermoplastic material into a mold cavity, and then cooling the cavity to solidify the resin. In the case of forming microchannel structures, a mold having the negative pattern of the desired channel structures must be created. Conventional tooling methods can be used to create molds for channels having dimensions as small as about 1 mm. Typically, enclosed microchannels are desired for the final structure. A common method for enclosing microchannel structures formed in plastics is to join a base and cover substrate using sonic welding. In addition, certain adhesives can also be used to join the base and cover substrates.

It has become desirable to create microchannel structures having capillary dimensions, *i.e.*, having dimensions ranging from less than 1 micron to upwards of 1 mm. These structures are of interest for manipulating very small fluid volumes through the application of electric fields to perform electrofluidics, *i.e.*, the movement of fluids in microchannels utilizing electrokinetic flow, that is, electrophoresis and/or electroosmotic flow (EOF). Electrophoresis is the movement of individual charged particles or molecules in response to the application of an electric field to an ionic solution. Electroosmotic flow is a bulk fluid flow (individual ions plus solvent molecules) that also results from the application of an electric field to an ionic solution. The extent of the bulk fluid flow is a function of the charge on the wall of the channel, as well as the viscosity of the solution. Both EOF and electrophoresis can be used to transport substances from one point to another within the microchannel device.

To create microchannels having capillary dimensions, photolithography in silicon or glass substrates has been employed. *See, e.g.*, U.S. Pat. No. 4,908,112, U.S. Pat. No. 5,250,263. In

the case of fused silica, these structures can be enclosed by anodic bonding of a base and cover substrate.

Although microchannel structures of such materials have been produced, it would be much more economical, and therefore desirable, to produce structures of capillary dimensions in polymeric materials or plastics. However, the conventional methods for forming and enclosing channels in plastic do not provide the accuracy and precision required for structures of capillary dimensions. For example, when using sonic welding, heating and deformation may occur in the channel regions. When the edges of a sonic weld are uneven, poor electrofluidic performance may result. Furthermore, sonic welding of highly defined intersections of capillary dimensions is not easily accomplished with adequate fidelity. Similarly, with conventional adhesive methods, the adhesive material may flow into and plug the channels.

Thus, there is interest in the development of new methods of fabricating polymeric microstructures, specifically in new methods of sealing the cover and base plates together, where such new methods do not result in deformation or filling in of the microchannels enclosed in the structure. Ideally, such methods should be simple and readily reproducible so as to be suitable for large scale manufacturing.

U.S. Pat. No. 5,376,252 to Eckström *et al.* describes a process for creating capillary size channels in plastic using elastomeric spacing layers. Öhman International Patent Publication WO 94/29400 describes a method for producing microchannel structures involving the application of a thin layer of a thermoplastic material to one or both of the surfaces to be joined, then joining the surfaces and heating the joined parts to melt the thermoplastic bonding layer.

SUMMARY OF THE INVENTION

Methods are provided for the fabrication of polymeric microchannel structures having enclosed microchannels of capillary dimension. The microchannel structures are constructed of a base plate and a cover, sealed together. Microchannel structures having walls of a plastic material are formed in a generally planar surface of at least the base plate. The cover has at least one generally planar surface, and the microchannel structures are enclosed by bonding the planar surfaces of the cover and the base plate together. The microchannel structures according to the invention find use in a variety of applications, particularly in electrofluidic applications.

Approaches to sealing the cover and base plate according to the invention include thermal bonding of the base plate and cover surfaces, and use of a bonding material between the base

plate and cover surfaces. Suitable bonding materials include elastomeric adhesive materials, and activatable bonding materials, including liquid curable adhesive materials and thermo-melting adhesive materials.

The thermal bonding approach can be employed where the apposing planar surfaces of the base plate and the cover are made of similar polymeric materials. Generally, in this approach, the planar surfaces of the base plate and the cover are aligned and confined to a mechanical fixture, in which they are progressively heated under pressure to a temperature 2 - 5 °C above the glass transition temperature of the polymer. In this first step, small irregularities in the surfaces accommodate to each other, while maintaining the physical integrity of the channels. Then, the temperature is maintained above the glass transition temperature of the polymer for a time sufficient to allow the polymer molecules to interpenetrate the two surfaces and create a morphological bonding. Above the glass transition temperature the molecules have sufficient entropy to entangle and orient in the surfaces of the two plates. In a final step of the bonding process the temperature is slowly reduced in order to maintain a stress free interface that provides a stable assembled microchannel structure.

Bonding materials can be employed where the apposing planar surfaces of the base plate and the cover are made either of similar or of different materials.

In approaches employing thermo-melting adhesives, the adhesive formulation includes medium molecular weight components that upon heating melt and diffuse into the two apposed surfaces, interpenetrating the two surfaces and creating a stable interface for the assembled microchannel structure. Suitable thermo-melting adhesives are usually formulated with chemistries that provide secondary bond interactions (*e.g.*, hydrogen bonding, Van der Waals forces, and hydrophobic forces) between the surfaces being bonded and the adhesive.

In approaches using liquid curable materials, one of the planar surfaces (usually of the cover), is coated with a layer or film of a liquid, curable adhesive material. The fluid layer is then rendered non-flowable, after which the coated surface is contacted with the apposing planar surface (usually of the base plate in which the microchannels have been formed). Then the curable adhesive material is cured to seal the surfaces together, forming the enclosed microchannel structure.

In approaches employing elastomeric bonding materials, the adhesive layer or film is a rubber or elastomer material (*e.g.*, natural and synthetic rubbers, polyurethane, polysulfides and silicones). The elastomeric bonding material can be applied in solution, as an emulsion, or in

formulations of two reactive components. Elastomeric bonding materials can be used in contact adhesive formulations, in which one or more small molecule components are admixed to provide tack; the do not require application of pressure to establish bonding. Or, elastomeric bonding materials can be used as pure elastomers to provide closure of the microchannel structures and to seal small irregularities in the generally planar apposed surfaces by application of pressure to exploit the compressibility of the elastomeric materials.

In some preferred methods according to the invention, the bonding process results in interpenetration into the two apposed planar surfaces, providing a stable sealed interface between the base plate and cover. Where a bonding material is used, a thin film or layer of the bonding material at the interface results.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagrammatic sketch in plan view of a base plate having a microchannel structure on one surface, which may be bonded to a cover according to the invention.

Fig. 2 is a diagrammatic sketch in sectional view thru the base plate of Fig. 1 at 2-2.

Fig. 3 is a diagrammatic sketch in plan view of a cover having a film of a bonding material on one surface, which may be bonded to a base plate according to the invention.

Fig. 4 is a diagrammatic sketch in sectional view thru the cover of Fig. 3 at 4-4.

Fig. 5 is a diagrammatic sketch in perspective view of a closed microchannel device fabricated by apposing and bonding the cover of Figs. 3, 4 onto the base plate of Figs. 1, 2 according to the invention.

Fig. 6 is a diagrammatic sketch in sectional view thru the microchannel device of Fig. 5 at 6-6.

Fig. 7 shows results of electrophoretic separation of fragments in the *HAE III* digest of ΦX174 RF DNA in a sealed plastic PMMA/Mylar™ microchannel structure.

Fig. 8 shows results of electrophoretic separation of fragments in the *HAE III* digest of ΦX174 RF DNA in a sealed plastic PMMA/PDMS microchannel structure.

Fig. 9 provides results of electrophoretic separation of single stranded DNA fragments having sizes from 50 to 500 bases at 50-base intervals in a thermobonded PMMA/PMMA microchannel structure.

Fig. 10 provides results of electrophoretic separation of single stranded DNA fragments having sizes from 35 to 500 bases in a PMMA/PMMA microchannel structure bonded using a thermally activated polymerizable bonding material.

DETAILED DESCRIPTION

The invention is now described in further detail, beginning with a description of the microchannel structures that are constructed according to the methods of the invention, followed by a discussion of the methods themselves.

The microchannel structures produced by the subject methods include at least one enclosed polymeric microchannel of capillary dimension. Thus, the structure may comprise a single enclosed microchannel or a network of interconnecting or separate microchannels having a variety of different configurations. Although the subject microchannels are enclosed, the enclosed channels will comprise at least one means of introducing liquid into the internal volume of the channel.

A microchannel of "capillary dimension", as that term is used herein, has cross-sectional dimensions that provide for capillary flow along the channel; usually a wider cross-sectional dimension of the channel is in the range about 50 μm to 750 μm, usually from about 100 μm to 500 μm and more usually from about 100 μm to 250 μm; a narrower cross-sectional dimension (usually the depth of the channel) can be somewhat smaller.

A "polymeric microchannel", as that term is used herein is a microchannel in which at least the inner surfaces of the microchannel walls, *i.e.*, that surface that contacts the liquid that is transported in the channel when in use, is of a polymeric material, where the thickness of the polymeric material will be at least about 1 μm, usually at least about 5 μm, and more usually at least about 50 μm, where the thickness may be as great as 5 mm or greater.

The structures may take a variety of different shapes; they may, for example, be including disc-like or card-like, and they may be layered or laminated "sandwich" structures. Representative shapes for such structures are further described in, for example, U. S. Patent Applications Serial Nos. 08/615,642 (A-63053-4), 08/853,661 (A-62852-2), 08/715,338 (A-62854) and 08/690,307 (A-62855).

In general, the microchannel structures according to the invention are constructed of two parts, each having at least one generally planar surface, sealed together so that the generally planar surfaces are apposed. One part is referred to as a base plate, and the other is referred to as

a cover. The planar surface of the base plate includes one or more microchannels, while the planar surace of the cover may or may not include one or more microchannels. The cover may be a more or less rigid plate, or it may be a film, and the thickness of the cover may be different for materials having different mechanical properties. Usually the cover ranges in thickness from at least about 200 μm, more usually at least about 500 μm, to as thick as usually about 5 mm or thicker, more usually about 2 mm. The cover substrate may be fabricated from a single material or be fabricated as a composite material. In some embodiments the cover is of a plastic material, and it may be rigid or elastomeric.

Both the base and cover substrates can be fabricated using any convenient methodology, such as molding, casting, extrusion sheet forming, calendaring, thermoforming, and the like. Suitable base and cover substrates for use in the subject invention are further described in U.S. Pat. Applications Serial Nos. 08/615,642 (A-63053-4), 08/853,661 (A-62852-2), 08/715,338 (A-62854) and 08/690,307 (A-62855).

Any of a variety of microchannel patterns, device shapes, and substrate materials can be used to construct and assemble the components of the microfluidic systems according to the invention, so long the device includes at least a generally planar base plate containing microchannels constructed of a plastic material. For example, a base plate and cover plate constructed of a plastic material can be bonded together directly (for example by thermal bonding), or by use of an adhesive layer. Or, a base plate constructed of a plastic material, in which the microchannels are formed, can be covered with a glass plate to enclose the channels, and sealed with an elastomeric film of, for example, a silicon or polyurethane elastomer. Glass provides improved dissipation of heat and better optical properties, as compared with plastic. Or, the device can be formed as a laminate (sandwich structure).

Construction of microchannel structures by bonding a base plate and a cover according to the invention will now be further described by reference for illustrative purposes to Figs. 1 - 6, in which Figs. 5 and 6 show an assembled microchannel device **10** made by bonding a base member or plate **12**, shown in plan and sectional views in Figs. 1 and 2 to a cover member or cover **14**, shown in plan and sectional views in Figs. 3 and 4, using a bonding material **16**. As will be appreciated, the drawings of the exemplary microchannel structures are not to scale and, in particular, certain dimensions (for example the thicknesses of the base plate, the cover, and the bonding material layer; and the sizes of the microchannels and reservoir holes) are shown in extremely exaggerated scale.

Base or substrate **12** has a planar surface **13** in which a microchannel structure is formed, including intersecting linear microchannels **21**, **23**. At the ends of the channels holes **22**, **24**, **26**, **28** are bored through, to provide reservoirs for fluids to be moved within the channels. Techniques for forming the microchannel structure in the base plate are disclosed, for example, in U.S. Patent Application Ser. No. 08/853,661 (A-62852-2). The microchannels as formed in the base plate are open, that is, absent a cover apposed to the channel-bearing surface **13** of the base plate, the microchannels are not fully enclosed.

Cover **11** has a generally planar surface **15**, apposable onto the channel-bearing surface **13** of planar member or base plate **12**, onto which a thin film **16** of a bonding material is applied. Microchannel device **10** is formed by apposing the surfaces **13**, **15** with the bonding material between them. As a result, the microchannels **21**, **23** are closed, having three walls formed in the base plate surface **13**, and a fourth wall formed by the cover **11**, with the bonding material film **16** constituting the surface of the fourth microchannel wall.

Reservoirs formed as described above are open on a surface of the base plate opposite the surface apposed to the cover. Other constructions may alternatively be employed for providing reservoirs. For example, holes can be bored only partway through the base plate at the ends of the channels, so that the reservoirs are not open on the opposite surface of the base plate; and holes can be bored or cut through the cover, aligned with the reservoirs. Liquids can be added to reservoirs formed in this manner can by filling through the holes in the cover, rather than from the opposite side.

In a method employing a curable bonding material according to the invention, a bonding material is applied onto a planar surface **15** of the cover material **14** to form a layer or film **16**. The bonding material may be, for example, a fluid curable adhesive, or a fluid component reactive with the cover material **14** and/or with the base plate material **12**, a meltable adhesive film, or a cured elastomeric film that provides physical or chemical characteristics to bind to the base plate in which at least one microchannel is formed.

Some care must be taken to apply the bonding material as a layer or film that is sufficiently thick and uniform to ensure that a continuous strong bond can form between the cover and the base plate at all points adjacent all the microchannels; and not so thick that too much of the bonding material adjacent the microchannels is displaced into the channels, distorting the channel shape or dimensions. An ideal thickness for the bonding material layer or film will accordingly be different for different bonding materials. In practice, generally, the bonding

material usually is applied to a thickness at least about 0.5 μm, in some embodiments at least about 1 μm, and in still other embodiments at least about 2 μm.

The bonding material layer or film may be applied to the surface using any convenient means suitable for application of a fluid layer to the surface of substrate. Such means finding use in the subject method therefore include: spin coating, dip coating, knife coating, drawing, rolling, mechanical spraying, atomization, patterned discharge, stamping, silk screening, lamination and the like, with the particular method employed being at least partially dependent on the nature of the substrate, *e.g.*, patterned discharge, stamping and lamination techniques being suited for use with flexible substrate materials.

As mentioned above, in some embodiments the apposed surfaces of the cover and base plate are bonded together according to the invention using a liquid curable adhesive. In these embodiments the adhesive is applied to one surface as a thin film. Accordingly, suitable fluid curable adhesives are flowable, having a viscosity in the range about 50 cp to 15,000 cp, usually about 50 cp to 10,000 cp, and more usually about 100 cp to 5,000 cp, where the viscosity is the viscosity of the material as measured at a temperature between about 15 °C and 50 °C.

Any of a variety of bonding materials can be useful in constructing microchannel structures according to the invention. Curable bonding materials can be particularly useful.

Curable bonding materials include materials applicable to all or a part of the substrate surface as a layer, coating, film, *etc.*, which upon application of energy result in formation of a durable stable interface material between the cover and the base materials. The durable stable interface material can be formed by covalent bonding, or interpenetration of the surface materials, or strong physical interaction, or by some combination of these. The energy can be one or a combination of heat, light, or other radiation including infrared or microwave radiation, for example; electron or other particle beam, and the like.

In some embodiments, where the movements of fluids or the progress of reactions within the microchannels are to be detected by means of light transmitted from the sample materials within the microchannel structure out through the cover or through the base plate, certain optical requirements must be met. Preferred modes of light detection may be based for example on UV and visible, luminescence and fluorescence responses of the sample material to incident radiation. For example, any material used in fabricating the cover, the base plate, or the bonding material on an enclosing wall of the microchannel should have good optical transmittance, generally allowing at least about 50 %, in some embodiments at least about 20 %, and in still other

embodiments at least about 10 % transmittance. And, for example, any material that is to be used in the field of fluorescence detection and through which light passes should have sufficiently low fluorescence in the detected bandwidths so that background fluorescence does not interfere with detection of the signal from the sample material.

Curable bonding materials finding use in the invention include polymerizable adhesives and activatable adhesives.

Polymerizable adhesives are those adhesives made up of polymerizable components including monomeric, oligomeric and low molecular weight polymeric compounds, where oligomeric and low molecular weight polymeric compounds present in the adhesive materials will generally have a molecular weight that does not exceed about 10^6, and usually does not exceed about 10^5, and more usually does not exceed about 10^3. The material may comprise one or a plurality of different types of polymerizable components, where when a plurality of different types of polymerizable components is present in the adhesive, the number of different components will generally not exceed 5 and will usually not exceed 3. The polymerizable components present in the polymerizable adhesives may be polymerizable by exposure to one or more of radiation (*e.g.*, electron beam, W radiation, microwave radiation, γ-radiation), and/or heat, as will be described in greater detail below. A variety of polymerizable components may find use in the subject materials, based on any mode of polymerization mechanism (including condensation, free radical, ionic, ring opening) where the components may be acrylic, methacrylic, cyanoacrylic, epoxide base, two-component epoxy adhesives, two-component urethane adhesives, and the like. Specific polymerizable components of interest include methylmethacrylate, ethylene glycol methacrylate, tetraethylene glycol methacrylate, cyano acrylate, uretheane prepolymers and diols, epoxy-containing prepolymers with amines, and the like. In the subject polymerizable adhesives, the polymerizable compounds will generally make up at least about 1 %, usually at least about 5 %, and more usually at least about 10 % by weight of the bonding material.

The polymerizable adhesive may include, in addition to the polymerizable components, one or more additional agents. One agent which may find use, depending on the particular adhesive employed, is a polymerization agent, where such agents include photosensitizers, photoinitiators, thermal initiators, and the like. Typical photosensitizers include the thioxantone derivatives; typical photoinitiators include benzophenone derivatives; and typical thermal initiators include the family of peroxy, perester, and azo initiators. When present, such

polymerization agents will generally not make up more than about 5 %, usually about 1 % and more usually about 0.5 % by weight of the bonding material.

Certain suitable polymerizable adhesive materials are commercially available, including for example (from Summers Corp., Fort Washington, PA): J91 (a single-component UV curable adhesive having a viscosity (uncured) of 250-300 cP; P92 (a single-component UV curable photopolymer having a viscosity (uncured) of 900-1400 cP; SK-9 (a single-component modified acrylate/methacrylate photopolymer having a viscosity (uncured) of 80-100 cP; DC-90 (a hybrid two-component UV sensitive cement having a viscosity (uncured) of 275-320 cP; EK-93 (a thixotropic two-component epoxy system having a viscosity of 25,000 cP; and (from Loctite Corp., Rocky Hill, CT): Depend 330 (a two-part mix acrylic thermocurable adhesive having a viscosity (uncured) greater than 10,000 cP.

Activatable adhesives finding use in the methods of the invention are those adhesives which include activatable polymeric compounds in combination with a carrier liquid. Depending on the particular adhesive, the polymeric compound may be dissolved in the carrier liquid (*i.e.*, the carrier liquid is a solvent for the polymeric compound) or dispersed in the carrier liquid, such that the adhesive material is an emulsion or suspension of the polymeric compound in the carrier liquid. The number of different activatable polymeric compounds in the activatable adhesives may range from 0 to 3, and usually range from 0 to2, more usually from 0 to 1. The activatable polymeric compounds will typically make up at least about 0 %, usually at least about 5 % and more usually at least about 10 % by weight of the adhesive.

An activatable polymeric compound is a polymeric compound that is capable of being treated so as to serve as an agent capable of bonding or sealing two substrates together. Activatable polymeric compounds include compounds comprising activatable functional groups, where illustrative activatable functional groups include groups that can form strong interactive forces with the surface of the base substrate. Specific applicable groups can be hydrogen bonding forming groups such as the urethane containing polymers or halogen containing polymers, such as polyvinyl chloride. The activatable polymeric component includes small molecular weight polymers that upon application of heat or pressure diffuse or penetrate into the surface of the base material, creating sufficient physical adhesion to maintain the integrity of the microchannel structures. Activatable polymeric compounds include rubbery elastomeric materials such as, for example, silicone gums and resins, the styrene-butadiene copolymers, polychloroprene, neoprene, and the nitrile and butyl containing elastomeric polymers; polylacrylics; polyurethanes;

polyamides; phenoxies; polyvinylacetals, and the like. Specific activatable polymeric compounds of interest include, for example: silicone gums, whereby upon application silicone polymers diffuse into the substrate surface, which can be thermocured after application to form crosslinked siloxane structures; urethane containing elastomers, which provide strong hydrogen bonding interaction with any polymer containing carboxyl groups, such as the acrylates; and nitrile containing polymers, which provide strong quasi-crossliniked intermolecular structures by dipole-dipole interaction of the nitrile groups.

The carrier liquid component of the activatable adhesive material may be any of a variety of different liquids, where the liquid is a liquid that is readily separable from the polymeric compound following application of the adhesive to the surface of the substrate. Illustrative liquids include solvents inert to the cover material that may be removed by evaporation, which may be carried out under low pressure. Carrier liquids will make up at least 50 %, usually at least 25 % and more usually at least 5 %, by weight of the adhesive material.

Other components which may be present in the activatable adhesive include reaction catalysts, thermal initiators, and photoinitiators; where such components are present, they will usually be present in an amount which does not exceed 5 %, more usually in an amount which does not exceed 1 %, by weight of the adhesive material.

Following application of the layer of fluid curable adhesive to the surface of the substrate, the applied layer will be rendered non-flowable. By non-flowable is meant that the applied layer is thickened so that the viscosity of adhesive layer will be increased to at least about 10^5 cP, in some embodiments at least about 10^6 cP and in still other embodiments at least about 10^7 cP. The manner by which the adhesive layer is rendered non-flowable will depend on the nature of the adhesive employed.

Thus, for polymerizable adhesives, the adhesive will be partially polymerized so that a sufficient percentage of the polymerizable components of the adhesive are polymerized to render the material suitably non-flowable and tacky. Generally, during partial polymerization at least about 50 %, usually at least about 75 % and not more than about 95 %, usually not more than about 90 % of the polymerizable components will be polymerized. Partial polymerization can be achieved using any convenient means, including radiation, heat, light, and the like.

For the activatable adhesives, the applied layer will be rendered suitably non-flowable by separating or removing the carrier liquid from the layer. Removal of carrier liquid may be

accomplished using any convenient means, including evaporation, which may be carried out under low pressure, and the like.

After the applied layer has been rendered non-flowable, the surface of the first substrate (*e.g.*, the cover) comprising the adhesive layer will then be contacted with the surface of the second substrate (*e.g.*, the base plate) in which the microchannel or microchannels are formed. To assist in ensuring sufficient contact, pressure may be employed, as convenient.

Following contact, the thickened adhesive will be cured, resulting in the bonding of the first and second substrates and the production of a sealed microchannel structure. The manner by which curing of the thickened adhesive is accomplished will depend on the type of adhesive employed. Thus, for polymerizable adhesives, the adhesive layer will already be partially polymerized and final curing may be accomplished by one ore more of exposure to radiation, heat, light and the like. Alternatively, for the activatable polymeric adhesives, the adhesive layer positioned between the first and second substrates will then be treated to activate the adhesive, where treatment could include exposure of the layer to radiation, heat, and the like, depending on the particular nature of the adhesive.

Where desired the above method may be further modified to include a substrate pretreatment step prior to the application of the adhesive to the substrate. Pretreatment steps that find use include exposure of the substrate, either the first or second substrate, to a cleaning and/or abrasion agent, etching agent, e.g plasma, corona, chemical, and the like, where such pretreatments provide for improved wettability of the surface of the substrate by the adhesive material.

EXAMPLES

The following examples are offered by way of illustration and not by way of limitation.

Example 1

This Example illustrates fabrication of a microchannel structure made up of a polymethylmethacrylate base plate bonded to a polymethylmethacrylate cover using a photocurable acrylate bonding material in a two-step curing process.

In this example, the bonding material is prepared as a blend of linear low molecular weight polymethylmethacrylate (MW = 10^5 - 10^6, 5 - 30 % w/w) dissolved in a mixture of methylmethacrylate monomer (60-95 % w/w), allylmethacrylate (1-5% w/w), and 0.5% w/w

2-hydroxy-2,2-dimethylactophenone (Darocure-1173, EM Industries) as a polymerization photoinitiator. Using spin coating tools, a thin layer (1 - 10 μm) of this bonding material is applied to the flat cover plate made of polymethylmethacrylate (PMMA). The coated cover is exposed for 10 - 30 seconds to a 15 watt fluorescent lamp at 1 inch from the surface. With this pre-curing step, the thin film has a non-flowable consistency, tacky toward the surface of a PMMA base plate with microchannel structures. The base plate is aligned and firmly positioned over the cover plate to form a base-to-cover sandwich, and 50 - 150 psi of pressure is applied to maintain the contact of base-to-cover and complete the bonding process by curing the acrylic interface under a 15 watt fluorescent lamp at 1 inch from the surface for 1-2 hours. The final cured interface provides a crosslinked, optically transparent cement between the cover and base plates, with good wall contact to the cover plate and open channels for analytical applications.

Example 2

This Example illustrates fabrication of a microchannel structure made up of a polymethylmethacrylate base plate bonded to a Mylar™ film cover using a thermally activated bonding material.

In this Example, the bonding material is a commercially-available thermally-activated adhesive. Photolithographic and electroforming techniques were used to prepare a mold, and injection molding techniques were used to prepare a microchannel base plate of an acrylic polymer (AtoHaas, Plexiglas™V825NA-100). The microchannel structure in this Example corresponds to two crossed linear channels of dimensions 2 cm and 5.5 cm in length respectively. The channels have a trapezoidal cross-section, with widths measuring about 120 μm and about 30 μm, and with an average depth about 40 μm. At the termini of the channels, holes 3 mm in diameter were drilled through the base plate to serve as buffer reservoirs. The channels were covered by thermal lamination of a 2 mil thick sheet of Mylar™ coated with a thermally-activated adhesive (MonoKote™, made by Top Flight Co.) at 105°C for 5 minutes. Electrodes of 76 micron diameter platinum wire were routed to each of the four reservoirs and terminated at one edge of the chip with a 4-prong 2.54 mm pitch KK® electrical heater (Waldon Electronics). No pretreatment or coating procedure was applied to the walls of the microchannel. The microchannel constructed this way has three walls whose surfaces are of

acrylic polymer, formed from the base plate, and a fourth wall whose surface is formed of the MonoKote adhesive.

Example 3

This Example illustrates separation of the fragments in the *HAE III* digest of ΦX174 RF DNA by capillary electrophoresis in a sealed plastic PMMA/Mylar™ microchannel structure fabricated as described in Example 2.

DNA separations were done in a sieving matrix consisting of 0.5% (w/v) hydroxyethylcellulose (HEC, MW 90,0000-105,0000), dissolved in 0.5X TBE with 2.5 μg/mL ethidium bromide. A sample of *Hae III* digest of ΦX174 RF DNA with fragments ranging in size 72 to 1354 base pairs previously diluted in run buffer was injected in the separation microchannel. After sample injection, separation of double stranded fragments was performed at an effective field strength of 190 V/cm. Fragment detection was performed through the PMMA base plate, using a fluorescence microscope (Olympus America) with photometer detection system (Photon Technology International). Excitation was derived from a deuterium lamp and delivered to the separation channel through a dichroic cube with 530 nm excitation filter, a 560-580 nm dichroic mirror, and a 590 nm long pass emission filter. Representative results of this separation are shown in Fig. 7 for an effective separation length of 4 cm with a total separation time of 2.6 minutes.

Example 4

This Example illustrates fabrication of a microchannel structure made up of a polymethylmethacrylate base plate covered with a film composite of a polyethylene (PE) and a polydimethylsiloxane (PDMS) elastomeric material in a two-step curing process.

In this Example, the cover film was prepared by applying a thin coat (about 100 μm) of Sylgard 184 (PDMS, Dow Corning) to a 2 mil thick polyethylene film (Barrier Films). The elastomeric layer of PDMS was pre-cured for 30 minutes in air at room temperature. The resulting pre-cured PE/PDMS composite was applied to a polymethylmethacrylate microchannel base plate made by injection molding as described in Example 2, and the PDMS allowed to cure further for 24 hours at room temperature. No pretreatment or coating procedure was applied to the walls of the microchannel. The microchannel constructed this

way has three walls whose surfaces are of acrylic polymer, formed from the base plate, and a fourth wall whose surface is formed of the PDMS elastomeric material.

Example 5

This Example illustrates separation of the fragments in the *HAE III* digest of ΦX174 RF DNA by capillary electrophoresis in a sealed plastic PMMA/PDMS microchannel structure fabricated as described in Example 4.

In this Example, the experimental parameters and conditions for the electrophoresis separation and detection of the *Hae III* digest of ΦX174 RF DNA fragments under non-denaturing conditions were as in Example 3. Results of the separation using the PMMA/PDMS microchannel structures are shown in Fig. 4. In this Example, separation of the eleven double stranded fragments was achieved in 5.0 minutes of total separation time.

Example 6

For this example, PMMA/PMMA microchannel structures were prepared using an injection molded microchannel base plate prepared generally as described in Examples 2 and 3. A flat PMMA plate injection molded using the same procedure was used as the cover plate to enclose the microchannel structure. Physical bonding between the base and cover plates was achieved using a thermobonding procedure carried out generally as follows. The microchannel base plate and the flat cover plate were mounted together in a mechanical fixture that allows plate heating and mechanical encasing of the two plates under pressure. For bonding, the fixture containing the microchannel cassette structure was heated to 104 °C at a rate of 1 °C/min in an oven. The temperature was then maintained 104°C for 2 hours. During this time, the two plate surfaces meltd and fused to each other. To complete the bonding, the temperature was reduced to room temperature at a rate of 1 °C/min. Then the fixture was opened and the PMMA/PMMA microchannel structure was removed. In the resulting microchannel containing cassettes, the channels are limited by four acrylic walls produced from the same acrylic injection molding resin. The walls are well-bonded, allowing the loading of viscous solutions under 100-200 psi pressure using a syringe adapted into the well opening of the microchannel.

As a demonstration of the separation of single stranded DNA fragments in such a thermobonded PMMA/PMMA structure, sieving gels were prepared as a solution of a linear

polymer (4.5 % by wt, MW=3 x 10^6) in 7 M urea and 1X TBE buffer. The separated fragments of DNA correspond to a Cy5 (indodicarbocyanine dye amidite, Biological Detection Systems, Inc.) labeled DNA Size Marker of single-stranded fragments ranging in size from 50 to 500 bases, separated by exactly 50 bases (Pharmacia). Electrophoresis was performed at 150 V/cm, with an effective channel length of 4 cm for 16 minutes. Detection was achieved using confocal fluorescence detection with He/Ne laser excitation (EX:633 nm) and emission at 670 nm. Figure 5 provides an example of the electrophoretic separation of single stranded DNA fragments obtained under these experimental conditions.

Example 7

This Example illustrates fabrication of a microchannel structure made up of a polymethylmethacrylate base plate bonded to a polymethylmethacrylate cover using a thermally curable bonding material in a one-step curing process.

In this example, the bonding material is prepared as a mixture of linear low molecular weight polymethylmethacrylate (MW = 10^5 - 10^6, 5 - 30 % w/w) in methylmethacrylate monomer (70-95 % w/w), and t-butylperoxypivalate (tBPP, Lupersol 11-Pennwalt) as a thermal polymerization initiator. Using spin coating tools, a thin layer (1 - 10 μm) of this bonding material is applied to a flat cover plate made of polymethylmethacrylate (PMMA). The base plate is aligned and firmly positioned over the cover plate to form a base-to-cover sandwich in a fixture, and pressure is applied using the fixture (50 - 150 psi) to maintain the contact of base-to-cover. The assembly of base-to-cover is carried out in a manner that avoids overflow of the channels with the bonding material. The bonding process is completed by curing the acrylic interface at 70 °C for 1-2 hours.

Example 8

This Example illustrates fabrication of a microchannel structure made up of a polymethylmethacrylate base plate bonded to a polymer film cover using a thermally curable bonding material in a one-step curing process.

In this Example a process similar to that of Example 6 is employed, by substitution of a cover film of 50-500 μm thickness for the PMMA cover plate. Films made of hydrocarbon based polymers (*e.g.*, low density polyethylene, amorphous polypropylene), fluorinated polymers (*e.g.*, polytetrafluoroethylene), or copolymers or blends of such polymers are

suitable for this process and provide added optical transparency required for spectroscopic (fluorescence and UV) detection through the cover. Chemical bonding of the acrylic base to the cover film is carried out generally as described above for the acrylic flat cover. In this format, the application of the bonding material to the cover film can be carried out manually, for example using a fine paint brush or rollers, or automatically, for example using roll coaters or float coaters.

Example 9

In this example, the bonding material is prepared as a blend of linear low molecular weight polymethylmethacrylate (MW = 10^5 - 10^6, 5 - 30 % w/w) dissolved in a mixture of methylmethacrylate monomer (60-95 % w/w), allylmethacrylate (1-5% w/w), and 0.5% w/w 2-hydroxy-2,2-dimethylactophenone (Darocure-1173, EM Industries) as a polymerization photoinitiator. Using spin coating tools, a thin layer (1 - 10 μm) of this bonding material is applied to the flat cover plate made of polymethylmethacrylate (PMMA). The coated cover is exposed for 10 - 30 seconds to a 15 watt fluorescent lamp at 1 inch from the surface. With this pre-curing step, the thin film has a non-flowable consistency, tacky toward the surface of a PMMA base plate with microchannel structures. The base plate is aligned and firmly positioned over the cover plate to form a base-to-cover sandwich, and 50 - 150 psi of pressure is applied to maintain the contact of base-to-cover and complete the bonding process by curing the acrylic interface under a 15 watt fluorescent lamp at 1 inch from the surface for 1-2 hours. The final cured interface provides a crosslinked, optically transparent cement between the cover and base plates, with good wall contact to the cover plate and open channels for analytical applications.

Example 10

This Example illustrates fabrication of a microchannel structure made up of a polymethylmethacrylate base plate bonded to a polymethylmethacrylate cover using a thermally curable bonding material using a one-step curing process.

A chemical bonding material was prepared as a mixture of linear low molecular weight polymethylmethacrylate (Mw = 10^5 - 10^6, 15% w/w) in methylmethacrylate monomer (85 % w/w), and t-butylperoxypivalate (tBPP, Lupersol 11, Penwalt) as a thermal polymerization initiator.

The microchannel structure was prepared from a base plate in which microchannels were formed, with recess wells bored partway through the base plate at the ends of the channels. The cover plate is provided with 2 mm diameter holes that align with the recess wells at the ends of the channels when the cover plate and base plate are apposed. The base plate and cover plate were made by injection molding from a polymethylmethacrylate resin (AtoHaas V825-NA100). After careful cleaning of the bonding surfaces with a surfactant containing solution, the curable bonding solution prepared as described above was applied onto the cover plate using standard spin coating equipment at 2000 rpm for 5 sec. The base plate was then carefully aligned onto the coated cover, with the apposing generally planar surfaces face-to-face. The sandwich structure assembled this way was then placed in a bonding fixture, configured to apply uniform pressure throughout the assembled structure. The interface layer is allowed to cure between the two plates under pressure of 40 psi in the fixture at 70 °C in an oven for two hours. After this curing process, the fixture containing the structure is cooled, and then the bonded microchannel structure is removed from the fixture in its final functional form.

The channel pattern used in this Example has a crossed-channel configuration, with reservoir wells at the ends of the channels. The shorter channel has a segment of length 0.4 cm from the well to the intersection on one side, and a segment of length 1.0 cm from the well to the intersection on the other side. The longer channel has segments of lengths 1 cm and 5 cm respectively from the well to the intersection. The channel cross-section has an asymmetrical trapezoidal shape of about 40 μm at the bottom of the channel and about 100 μm at the top of the channel. The channel depth after bonding was 35 μm as measured in scanning electron micrographs .

Example 11

This Example illustrates separation of a DNA ladder under denaturant conditions by capillary electrophoresis in a sealed plastic PMMA/PMMA microchannel structure constructed as decribed in Example 8.

Without any preconditioning of the microchannel surface, the microchannels were filled with a 5 % linear polyacrylamide (MW = 2 - 3 × 10^6) solution in 1×TBE buffer and 7 M urea using a pressurized syringe loading device with liquid tight connections to the well at the end of the long arm of the long channel. A solution of GeneScan 500 DNA ladder with tagged TAMRA label was electrokinetically injected into the channel cross-section from the short arm of the short

channel. After a sample plug was formed at the channel intersection, a separation voltage of 200 V/cm was applied between the two wells of the long channel using platinum wire electrodes (76 μm). Detection of the separating bands in the longer segment of the longer channel was performed using a fluorescence microscope (Olympus America) with photometer detection system (Photon Technology International). Excitation was derived from mercury lamp and delivered to the separation channel through a dichroic cube with 535-550 nm excitation filter, a 560-580 nm dichroic mirror, and a 570 nm long pass emission filter.

Representative results of separations made in this way are shown in Fig. 10. The effective length of the separation is 4 cm, and separation was achieved in a total separation time of 7.5 minutes. The GeneScan 500 ladder sample contains 16 DNA fragments with different number of incremental bases from each fragment. Fragment separation of fragments differing by 10 bases are labeled in the electropherogram of Fig. 10. Normalized single base resolution was calculated at between 0.3 and 1.2 for the 35 to 500 bases separation range.

It is evident from the above results and discussion that improved methods for fabricating polymeric microchannel structures suitable for use in electrofluidic applications are provided. By using the subject methods, cover and base plate components of the structures can be sealed together without deformation, partial or complete clogging of the enclosed microchannels.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS

What is claimed is:

1. A method for constructing an enclosed microchannel structure having at least one microchannel, the method comprising providing a base member of a plastic material having at least one generally planar surface, providing a cover member having at least one generally planar surface, forming at least one microchannel in at least one of said planar surfaces, apposing said planar surface of said cover member and said planar surface of said base member and causing formation of a stable interface between said apposed planar surfaces.

2. The method of claim 1 wherein said planar surface of said cover member is fabricated of a plastic material and wherein said step of causing formation of a stable interface comprises pressing said apposed surfaces together and heating said members for a time and to a temperature sufficient to bond said surfaces together.

3. The method of claim 1 further comprising a step prior to said apposing step of applying a bonding material to at least one of said planar surfaces.

4. The method of claim 3 wherein said bonding material comprises an elastomeric adhesive material.

5. The method of claim 3 wherein said bonding material comprises a thermo-melting bonding material and wherein said step of causing formation of a stable interface comprises heating said bonding material to a temperature and for a time sufficient to melt said bonding material and then cooling said bonding material between said apposed surfaces to permit the bonding material to harden.

6. The method of claim 3 wherein said bonding material comprises an activatable bonding material.

7. The method of claim 3 wherein said bonding material comprises a curable bonding material.

8. The method of claim 3 wherein said bonding material comprises a polymerizable bonding material.

9. The method of claim 7 wherein said curable bonding material is applied to said surface in a flowable state and wherein said method further comprises the step, following the step of applying said bonding material to at least one of said planar surfaces and prior to the step of apposing the surfaces, of partially curing said curable bonding material to a non-flowable state.

10. The method of claim 8 wherein said bonding material further comprises a polymerization initiator.

11. The method of claim 10 wherein said polymerization initiator comprises a photoinitiator and wherein said step of causing formation of a stable interface comprises exposing said bonding material between said apposed surfaces to light at a wavelength and intensity and for a time sufficient to cause polymerization.

12. The method of claim 10 wherein said polymerization initiator comprises a thermal initiator and wherein said step of causing formation of a stable interface comprises heating said bonding material between said apposed surfaces to a temperature and for a time sufficient to cause polymerization.

13. The method of claim 1 wherein said cover member comprises an elastomeric material.

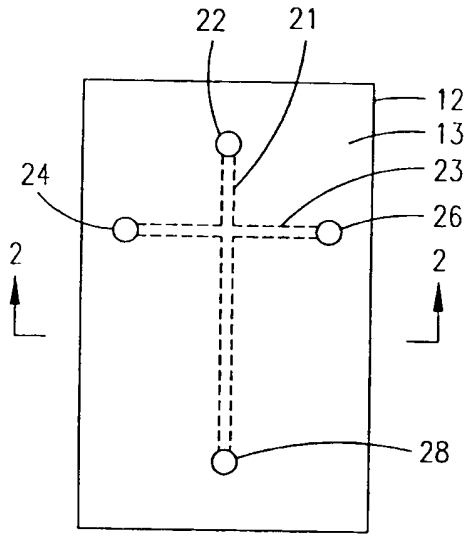


FIG. 1

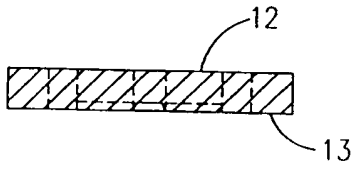


FIG. 2

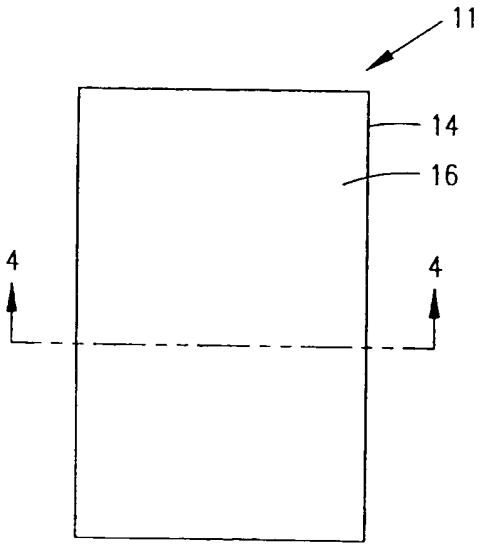


FIG. 3

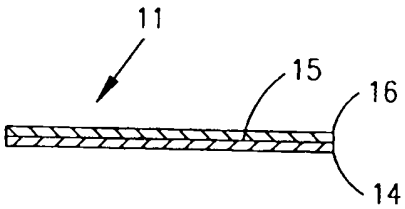


FIG. 4

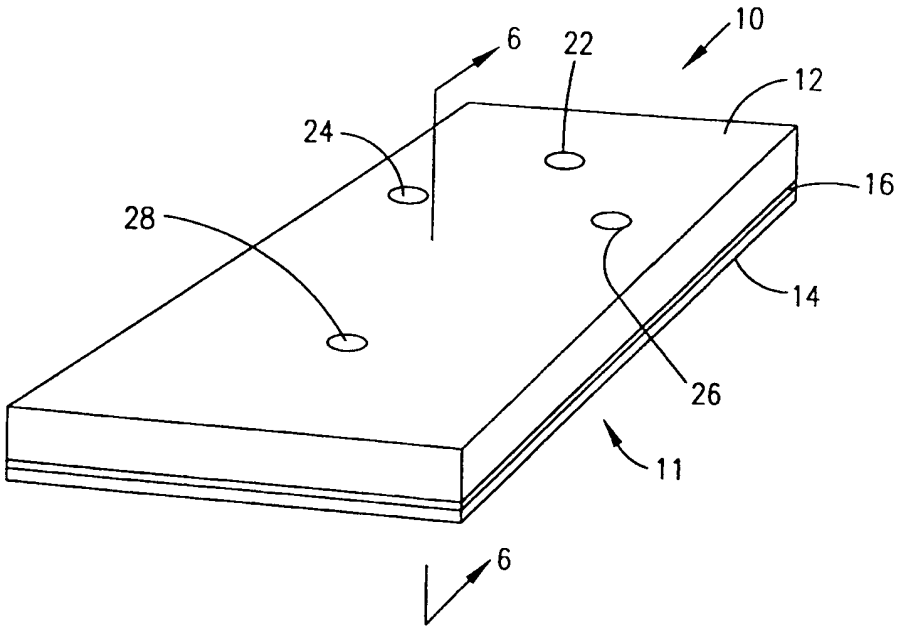


FIG. 5

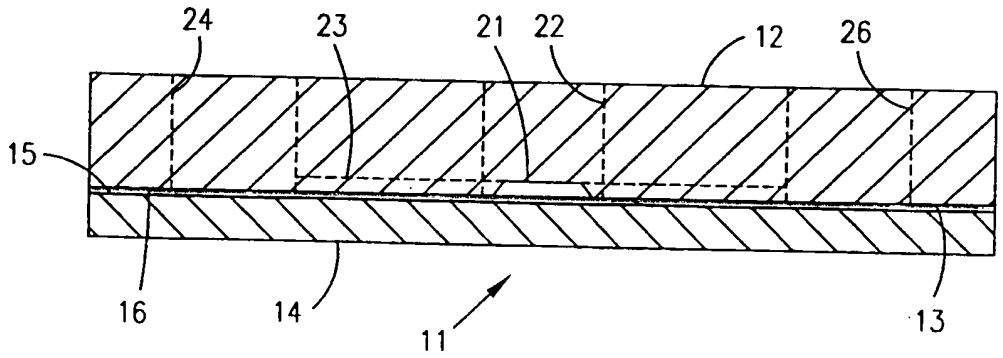


FIG. 6

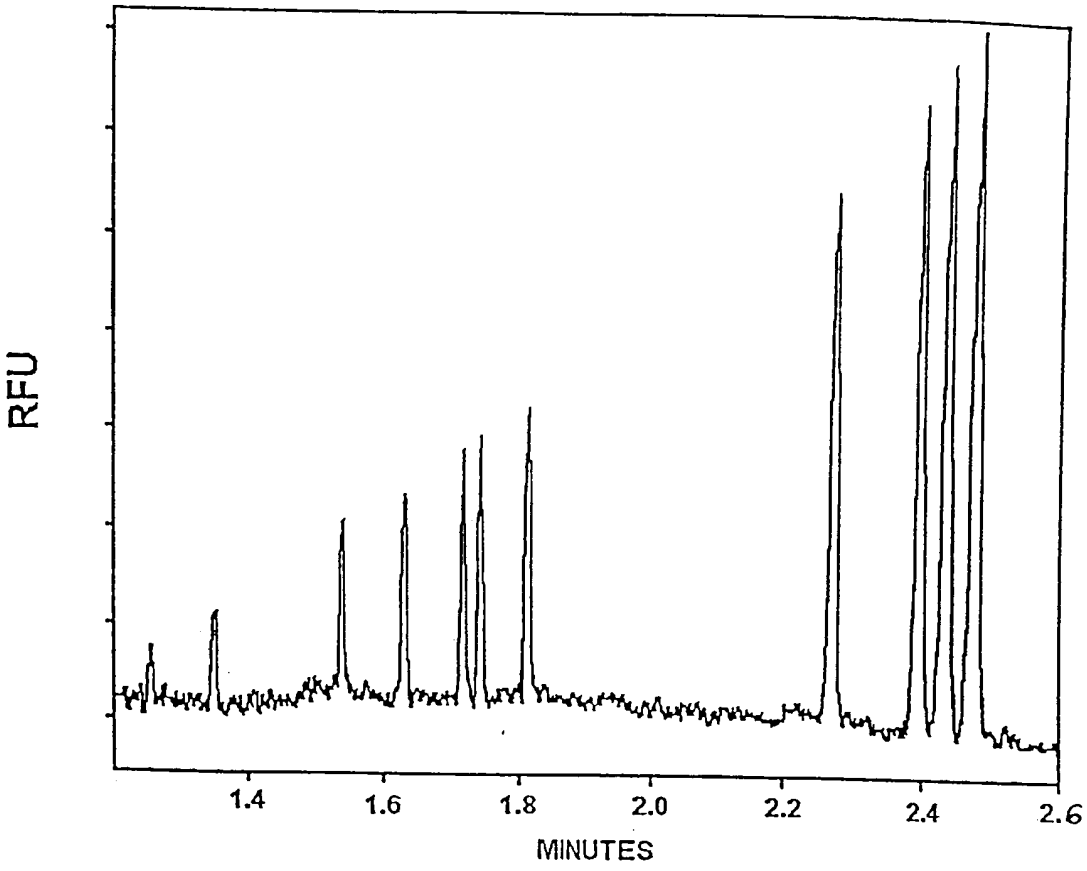

RFU
1.4
1.6
1.8
2.0
2.2
2.4
2.6
MINUTES

Fig. 7

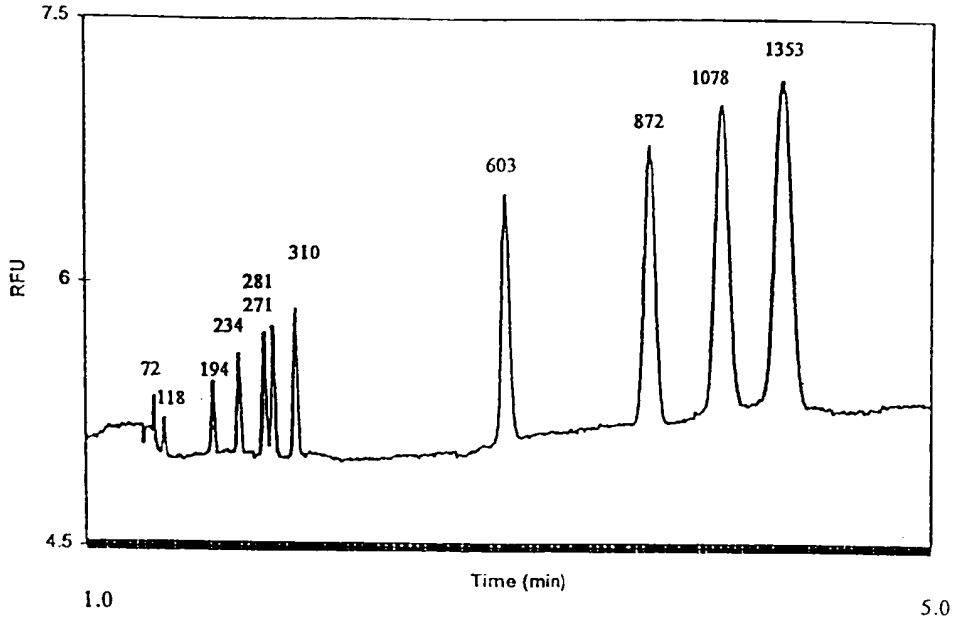

7.5
6
4.5
RFU
72
118
194
234
281
271
310
603
872
1078
1353
1.0
Time (min)
5.0

Fig. 8

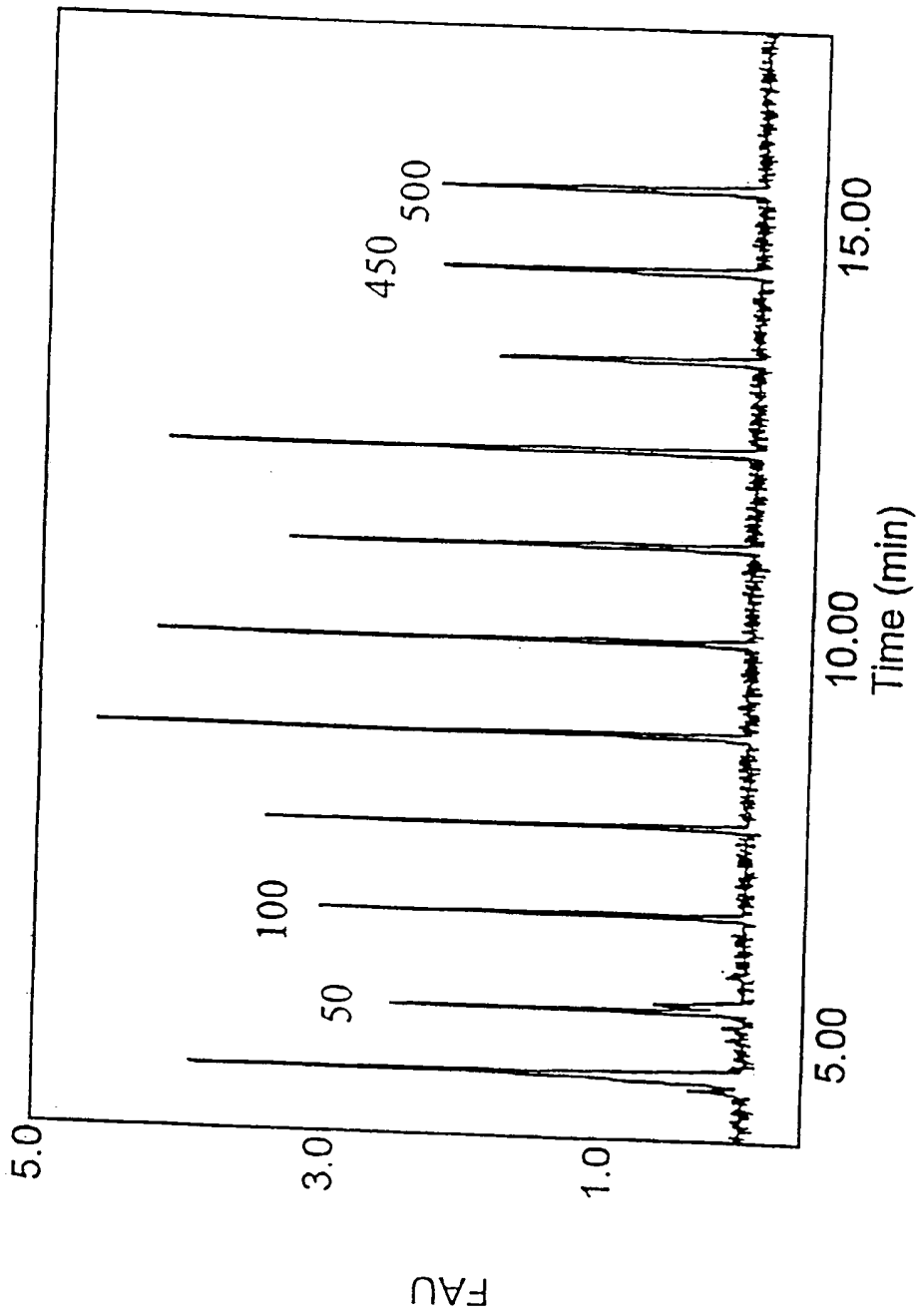


Fig. 9

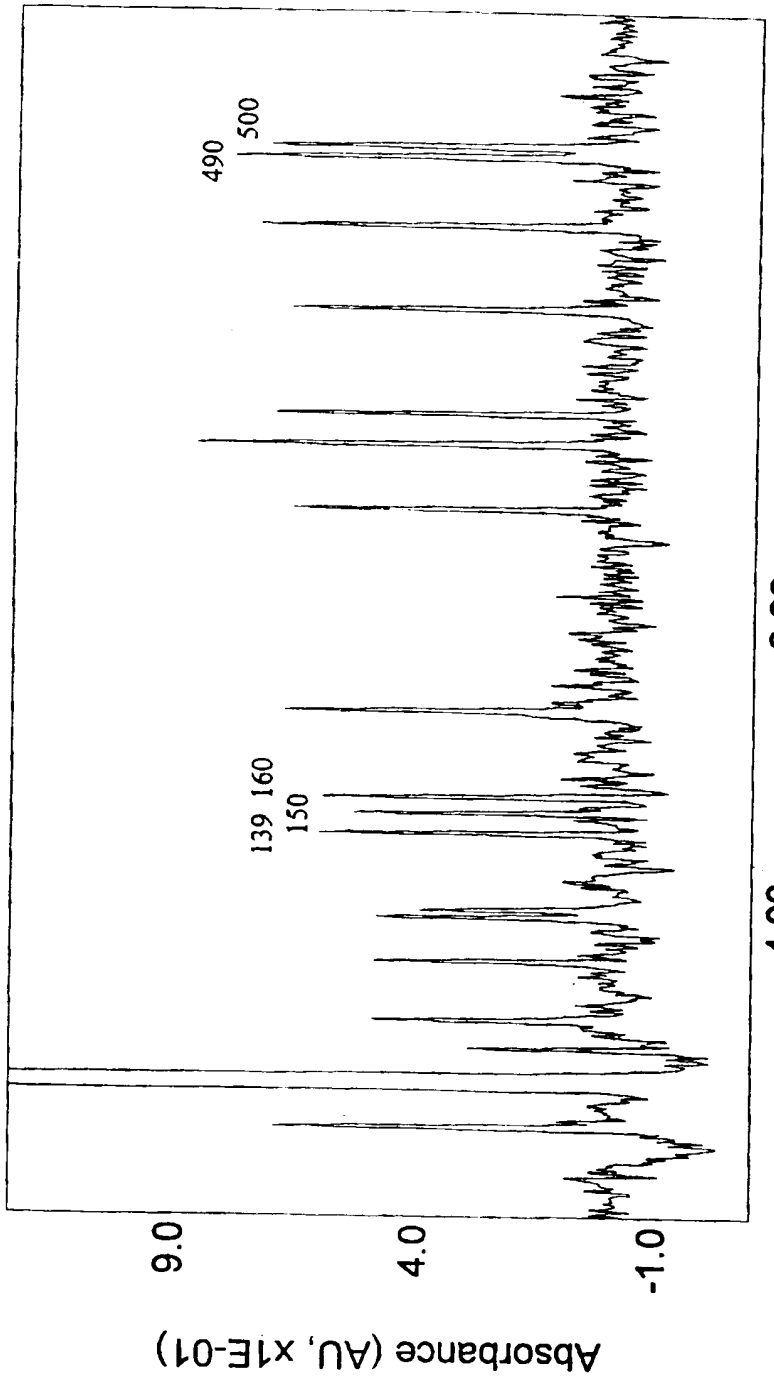

490
500
139 160
150
4.00
6.00
8.00
Time (min)
9.0
4.0
-1.0
Absorbance (AU, x1E-01)

Fig. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06689

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :G0IN 27/26, 27/447
US CL : USE 204/451,601
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : US 204/451-455, 601-605

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 4,908,112 (PACE) 13 March 1990 (13/03/90) see column 6, lines 24-55, column 7, lines 2-6, and column 7, lines 14-533.	1, 3, 4, 6 --- 2, 5, 7-13
X — Y	US 4,891,120 (SETHI ET AL) 02 January 1990 (02/01/90) see column 4, lines 24-30.	1, 3 --- 2, 4-13
X — Y	US 5,571,410 (SWEDBERG ET AL) 5 November 1996 (05/11/96) see column 15, line 65-column 16, line 4, column 18, lines 9-11, column 23, lines 10-20, and column 26, lines 19-41.	1, 2, 3, 5, 6 13 --- 4, 7-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search
22 JUNE 1998

Date of mailing of the international search report
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(81) **Bestimmungsstaaten:** CA, US, europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Veröffentlicht
Mit internationalem Recherchenbericht.
Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist; Veröffentlichung wird wiederholt falls Änderungen eintreffen.

(54) **Title:** OPTICALLY ACTIVE ELEMENT AND METHOD FOR THE PRODUCTION THEREOF

(54) **Bezeichnung:** OPTISCH AKTIVES ELEMENT UND VERFAHREN ZU SEINER HERSTELLUNG

(57) **Abstract**

The invention relates to optically active elements made of a plastic material that is transparent to electromagnetic waves and to a method for the production of said element. Said optical elements should have at least a reduced reflection on their boundary surfaces with the surrounding medium. In order to solve this problem, a microstructure should be formed directly at least in part on the surface of the elements. The widths or distances of the individual structural elements are smaller than the smallest wavelength of the electromagnetic waves influencing the element.

(57) **Zusammenfassung**

Die Erfindung betrifft optisch aktive Elemente aus einem für elektromagnetische Wellen transparenten Kunststoffmaterial sowie Verfahren zu deren Herstellung, wobei solche optischen Elemente an ihren Grenzflächen zum umgebenden Medium eine zumindest verringerte Reflexion aufweisen sollen. Zur Lösung dieses Problems soll unmittelbar auf der Oberfläche solcher Elemente zumindest teilweise eine Mikrostrukturierung flächenhaft ausgebildet werden und dabei die Breiten und Abstände der einzelnen Strukturelemente kleiner als die kleinste Wellelänge der mit dem Element zu beeinflussenden elektromagnetischen Wellen sein.

Optisch aktives Element und Verfahren zu seiner Herstellung

Die Erfindung betrifft optisch aktive Elemente aus einem für elektromagnetische Wellen transparenten Kunststoffmaterial sowie Verfahren zu deren Herstellung.

Bei optisch aktiven Elementen aus transparenten Materialien für elektromagnetische Wellen, im sichtbaren und nicht sichtbaren Wellenlängenbereich treten Probleme an den Grenzflächen dadurch auf, daß das einfallende Licht teilweise reflektiert und demzufolge entsprechende Lichtverluste in Kauf genommen werden müssen.

Aus dem Stand der Technik sind diesem Nachteil entgegenwirkend einige Maßnahmen bekannt, um die Reflexionsverluste zumindest zu verringern.

Zum einen werden hierfür zusätzlich dielektrische Interferenzschichten oder Interferenzschichtsysteme auf die Oberflächen aufgebracht, um den reflektierten Lichtanteil zu verringern. Dabei ist festzuhalten, daß diese Möglichkeit nicht in jedem Fall praktikabel ist und insbesondere bei den verschiedenen optisch aktiven Elementen, wie z.B. Prismen oder Fresnellinsen nicht ohne weiteres eingesetzt werden kann.

Außerdem treten eine Reihe von Beschränkungen auf. Insbesondere bei der Beschichtung von Kunststoffen kann die Haftung der Schicht auf dem Substrat kritisch sein, so daß sich die Schicht bei geringster mechanischer Belastung ablöst. Desweiteren ist die

Entspiegelung stark strukturierter Oberflächen wie z.B. Fresnellinsen oder -prismen mit Interferenzschichtsystemen beim gegenwärtigen Stand der Technik nur in Spezialfällen möglich. Die entspiegelnde Wirkung von Interferenzschichtsystemen hängt stark vom Einfallswinkel der Strahlung ab, was bei Anwendungen mit einem breiten Winkelspektrum problematisch ist.

Eine andere Möglichkeit, die auf die Erkenntnisse von Fresnel bezüglich der Reflexion und Reflexminderung aufbaut, ist in GB 1 529 021 beschrieben. Dabei werden in transparenten Platten aus Kunststoffmaterialien Ausstülpungen durch Prägen in solche Platten erzeugt, um die Lichtverluste an Bedachungen von Gewächshäusern, die normalerweise reflexionsbedingt auftreten, zu verringern.

In einer nicht vorveröffentlichten deutschen Patentanmeldung ist darüber hinaus eine Möglichkeit beschrieben, um eine Entspiegelungsschicht auf einem optisch transparenten Material zu erhalten. Dabei wird eine Trägerschicht auf mindestens einer Oberflächenseite mit einer eine Makrostruktur überlagernden Mikrostrukturierung versehen. Die Makrostrukturierung wirkt ähnlich wie eine Streuscheibe, hat aber den Nachteil, daß das Kontrastverhältnis verringert wird. Insbesondere dieser Nachteil sollte bei optisch aktiven Elementen vermieden werden, um die gewünschten Abbildungen nicht entsprechend zu verschlechtern. Die der Makrostrukturierung überlagerte Mikrostrukturierung hat wiederum lediglich die Aufgabe der Verminderung der Reflexion an der Oberfläche. Diese Lösung zielt auf die Beseitigung störender Reflexe und weniger auf die Erhöhung der Transmission. Die Makrostruktur dient der Umwandlung klar erkennbarer in

diffuse Reflexe; die Mikrostruktur reduziert die diffusen Reflexe, um den Kontrast zu verbessern. Für optisch aktive Elemente stellt ein diffuser Reflex im allgemeinen keine Verbesserung gegenüber einem klaren Reflex dar, weil die Lichtausbeute verringert wird und das Störlicht im optischen System verbleibt (besonders bei Objekten mit vielen brechenden Flächen) und den Kontrast verschlechtert.

Neben der Verringerung des Kontrastes ist die dort beschriebene Lösung ebenfalls nicht ohne weiteres an optisch aktiven Elementen einsetzbar, da eine vorbereitete Trägerschicht nicht immanenter Bestandteil eines optisch aktiven Elementes ist und demzufolge zwischen Trägerschicht und eigentlichem Substrat des optisch aktiven Elementes eine Grenzfläche mit den bekannten Nachteilen ausgebildet ist. Desweiteren können solche Trägerschichten nicht ohne weiteres auf komplex konturierte optische Elemente aufgebracht werden.

Außerdem können Haftungsprobleme, die auch bei den Interferenzschichtsystemen zu berücksichtigen sind, insbesondere bei den Kunststoffmaterialien auftreten.

Erläuternd soll weiterhin darauf hingewiesen werden, daß bestimmte optisch aktive Elemente, wie Linsen, Prismen, oder Lentikulare mit geringer Dicke und großer Öffnung nach dem von Fresnel entwickelten Prinzip durch entsprechende Oberflächenstrukturierung herstellbar sind. Bei solchen optischen Elementen ist deren Dicke zumindest über die optisch wirksame Fläche nahezu konstant. Dabei werden Wirkflanken verwendet, die konzentrisch bzw. parallel zueinander angeordnet sind und infolge der Ausbildung der Wirkflan-

ken entsprechende diese voneinander trennenden Störflanken vorhanden sein müssen, die mehr oder weniger Lichtverluste bedingen. Dabei gibt es bei solchen optischen Elementen solche mit einseitiger Strukturierung und der entsprechenden Gegenseite, die in diesem Fall normalerweise als Planfläche ausgebildet ist. Solche optischen Elemente können aber auch an beiden Seiten entsprechend mit Wirk- und Störflanken strukturiert sein.

Selbstverständlich treten auch bei solchen optischen Elementen beim Durchgang des Lichtes durch entsprechende optische Grenzflächen Reflexionen auf, die Intensitätsverluste und Kontrastverschlechterung bedingen und zusätzlich störende, visuell auffällige Reflexe verursachen können.

Insbesondere bei Fresnel-Linsen, die eine kurze Brennweite erreichen, treten die elektromagnetischen Wellen unter großen Winkeln (bis zu 70°) entweder durch die plane Fläche und/oder die vorhandenen Wirkflächen (infolge der Strukturierung) aus. Durch erhöhte Reflexion kann es eine zumindest teilweise Polarisation des Lichtes geben. Außerdem kann der Anteil an reflektiertem Licht an der nächsten Grenzfläche totalreflektiert werden bzw. durch Störflanken austreten. Durch die hohe Komplexität können all diese Möglichkeiten nicht berücksichtigt bzw. vorberechnet werden, so daß es zur Konzentration von Störlicht auf der optischen Achse bzw. auf konzentrischen Ringen (Abbildungsfehler), bei Fresnellinsen, kommen kann, deren Lage von den Abständen im optischen System abhängig ist.

Es ist daher Aufgabe der Erfindung, optisch aktive

Elemente zur Verfügung zu stellen, die an ihren Grenzflächen zum umgebenden Medium eine zumindest verringerte Reflexion aufweisen.

Erfindungsgemäß wird diese Aufgabe mit den Merkmalen des Patentanspruchs 1 gelöst. Mögliche Verfahren zur Herstellung solcher optisch aktiven Elemente sind mit den Merkmalen der Patentansprüche 7, 8 und 9 beschrieben. Vorteilhafte Ausgestaltungsformen und Weiterbildungen der Erfindung ergeben sich bei Verwendung der in den untergeordneten Ansprüchen genannten Merkmale.

Das erfindungsgemäße optisch aktive Element aus einem für elektromagnetische Wellen im sichtbaren und nicht sichtbaren Wellenlängenbereich transparenten Kunststoffmaterial ist dabei so gestaltet, daß zumindest die Oberflächenbereiche, an denen die unerwünschten Reflexionen vermieden werden sollen, unmittelbar auf der Oberfläche eine flächenhafte Mikrostrukturierung aufweisen, bei der die Abstände der einzelnen Strukturelemente jeweils kleiner als die kleinste Wellenlänge der mit dem Element zu beeinflussenden elektromagnetischen Wellen sind.

Mit einer solchen Ausbildung einer Mikrostrukturierung können die unerwünschten Reflexionen zumindest stark verringert werden, da ein nahezu kontinuierlicher Brechzahlübergang zwischen der Umgebung, in der Regel Luft, und der eigentlichen Oberfläche des optisch aktiven Elementes zu verzeichnen ist, da der Anteil an z.B. Luft innerhalb der Mikrostrukturierung in Richtung auf das optisch aktive Element kontinuierlich abnimmt. Dies wird durch die Gestaltung der Mikrostrukturierung begünstigt bzw. erreicht, in dem

das freie Volumen, das mit dem Umgebungsmedium (Luft oder ein anderes Gas) ausgefüllt ist, innerhalb der Mikrostrukturierung mit wachsender Tiefe kontinuierlich abnimmt.

Die Mikrostrukturierung kann dabei einmal periodisch ausgebildet sein und zum anderen können die einzelnen Strukturelemente einer solchen Mikrostrukturierung auch stochastisch verteilt auf den entsprechenden Oberflächenbereichen ausgebildet sein. Auch die Form der Strukturelemente kann stochastisch sein, z.B. Pyramiden mit einer Größenverteilung.

Dabei sollten bei linien- oder gitterförmigen periodischen Strukturen die Perioden zwischen den Strukturelementen und bei stochastischen Strukturen die einzelnen Strukturelemente und Abstände zwischen diesen, jeweils kleiner als die kürzeste Wellenlänge, der mit dem optischen Element zu beeinflussenden elektromagnetischen Strahlung sein.

Es können aber auch Mischformen von periodischer Ausbildung und stochastischer Anordnung Verwendung finden, wenn das Abstandserfordernis für die Strukturelemente zumindest überwiegend erfüllt ist.

Die Mikrostrukturierung sollte vorteilhaft auf der Oberfläche der optisch aktiven Elemente mit einer Tiefe bis zu maximal 1,5 µm, bevorzugt bis zu 1 µm ausgebildet sein, wenn z.B. im Wellenlängenbereich des sichtbaren Lichtes gearbeitet werden soll. Für Infrarotlicht kann die Struktur auch einige µm breit und tief sein.

Erfindungsgemäß können optisch aktive Elemente:

Prismen, Lentikulare, Strahlteiler, konkav- oder konvexgeformte Linsen, Zylinderlinsen, Fresnel-Spiegel, wenn als Rückseitenspiegel verwendet, oder Fresnellinsen sein. Dabei können solche Linsen, wie dies bei Fresnellinsen generell der Fall ist, auch mit anderen Strukturierungen versehen sein, der die erfindungsgemäß zu verwendende Mikrostrukturierung überlagert wird.

Dabei ist es sicher günstig, nicht nur die entsprechenden Wirkflanken, sondern auch die Störflanken solcher optisch aktiven Elemente erfindungsgemäß zu modifizieren.

Einflußgrößen für die spektrale Bandbreite der reflexionsmindernden Wirkung, sind im kurzwelligen Bereich wiederum die Abstände zwischen den einzelnen Strukturelementen bzw. die Breite der jeweiligen Periode und diese wird im langwelligen Bereich durch die Tiefe der Mikrostrukturierung begrenzt, das bedeutet, daß man für größere Wellenlängen tiefer prägen muß.

Ist die Strukturgröße im Vergleich zur Wellenlänge, für die das Bauelement eingesetzt wird, zu groß, tritt Beugung auf.

Die Mikrostrukturierung kann auf Oberflächen des jeweiligen optisch aktiven Elementes ausgebildet sein, in die das Licht ein- bzw. austritt, wobei in jedem Fall die reflexionsmindernde Wirkung auftritt.

Mögliche Kunststoffmaterialien für die Herstellung der erfindungsgemäßen optisch aktiven Elemente sind Polymethylmethacrylate, Polycarbonate sowie andere geeignete Polymere, die kostengünstig herstell- und

verarbeitbar sind. Dabei kann die Herstellung der erfindungsgemäßen optisch aktiven Elemente in der Form erfolgen, daß die Mikrostrukturierung bereits während des eigentlichen Herstellungsverfahrens solcher Elemente durch Spritzgießen oder Heißprägen ausgebildet wird. Hierfür können Werkzeuge verwendet werden, an deren Oberfläche ein Negativabbild der unmittelbar auf der Oberfläche des optisch aktiven Elementes auszubildenden Mikrostrukturierung ausgebildet ist. Dieses Vorgehen hat den Vorteil, daß keine zusätzlichen Verfahrensschritte für die Herstellung solcher optisch aktiven Elemente erforderlich sind, die bei anderen noch zu beschreibenden Verfahren, mit denen solche Elemente herstellbar sind, erforderlich sind. Beim Herstellen der erfindungsgemäßen Elemente durch Spritzgießen mit unmittelbarer und gleichzeitiger Ausbildung der Mikrostrukturierung in bestimmten Oberflächenbereichen ist es auch günstig, die Werkzeuge zusätzlich zu beheizen, um Spannungen im Kunststoffmaterial zu vermeiden und es dem eingespritzten Kunststoffmaterial durch ausreichende Fließfähigkeit zu ermöglichen, die erfindungsgemäß gewünschte Mikrostrukturierung auszubilden.

Dabei sollte das Temperaturverhalten des verwendeten Kunststoffes berücksichtigt werden, da zumindest die mit der Mikrostrukturierung zu versehenden Oberflächenbereiche ausreichend erweicht sein müssen.

Eine weitere Möglichkeit zur Herstellung der erfindungsgemäßen optisch aktiven Elemente besteht darin, die Mikrostrukturierung mittels eines Lasers auf der Oberfläche eines solchen Elementes auszubilden. Hierfür sind insbesondere Eximerlaser und in begrenzter Form auch NdYAG-Laser geeignet. Der Laserstrahl eines

solchen Lasers wird hierbei bevorzugt in zwei Ebenen abgelenkt über die Oberfläche, die mit der Mikrostrukturierung versehen werden soll, geführt, wobei zusätzlich eine Beeinflussung der Fokussierung des Laserstrahls erforderlich sein kann, wenn eine nicht ebene Fläche mikrostrukturiert werden soll. Dabei kann der Laserstrahl durch eine die Mikrostrukturierung vorgebende und entsprechend ausgebildete Maske auf die Oberfläche gerichtet werden, um die erforderlichen Abstände der einzelnen Strukturelemente zu sichern.

Die Laserstrahlbehandlung wirkt sich insbesondere bei kleinen Stückzahlen kostengünstiger aus, als dies bei dem anderen erwähnten Herstellungsverfahren der Fall ist.

Da die erfindungsgemäßen optisch aktiven Elemente keinen Materialverbund darstellen, sondern aus einem einzigen homogenen Kunststoffmaterial bestehen, treten die aus dem Stand der Technik bekannten Nachteile nicht auf, so daß ein Langzeiteinsatz auch bei stark schwankenden Temperaturen ohne weiteres möglich ist. Zusätzlich verfügen diese Elemente über eine ausreichende mechanische Festigkeit, verringern Falschlichtanteile und verbessern Kontrast sowie Lichtausbeute.

Nachfolgend soll die Erfindung beispielhaft beschrieben werden.

Als ein optisch aktives Element wird eine Fresnellinse aus einem PMMA mit dem Brechungsindex n = 1,496 (bei 500 nm) durch Heißprägen mit einem Fresnel-Prägestempel und einer Gegenplatte hergestellt. Es han-

delt sich dabei um eine Fresnellinse, die auf einer Seite die bekannte Struktur aus Wirk- und Störflanken aufweist, während die andere Seite planar ist. Auf der Gegenplatte, die die Form der planaren Oberfläche der Fresnellinse vorgibt, ist das negative Abbild der gewünschten Mikrostrukturierung ausgebildet.

Das PMMA wird in Form von Granulat, Pulver oder einem plattenförmigen Halbzeug auf den in der Prägevorrichtung untenliegenden Fresnel-Prägestempel aufgebracht und durch Heizung des Fresnel-Prägestempels sowie der Gegenplatte bis über die Erweichungstemperatur 108°C, typischerweise auf etwa 180°C, erwärmt. Durch definiertes Absenken der Gegenplatte mit einem Enddruck von etwa 10 MPa werden in den fließfähigen Kunststoff gleichzeitig die Fresnelstruktur auf der einen Seite und die entspiegelnde Mikrostruktur auf der anderen Seite eingeprägt. Nach einer Abkühlungsphase wird die Fresnellinse bei einer Temperatur unterhalb 98°C, der Grenztemperatur für die Formbeständigkeit des PMMA, entformt.

Die planare Seite der so hergestellten Fresnellinse zeichnet sich gegenüber einer planaren Fläche ohne Mikrostruktur durch eine verminderte Reflexion aus. Bei senkrechtem Lichteinfall werden durch die Fläche mit Mikrostruktur nur etwa 1,5 % des einfallenden Lichtes im sichtbaren Wellenlängenbereich reflektiert, während eine gewöhnliche planare Fläche etwa 4 % des einfallenden Lichtes reflektiert. Dadurch wird die Lichtausbeute in optischen Systemen verbessert und die Ausbildung störender Reflexionen wirksam unterdrückt.

Patentansprüche

1. Optisch aktives Element aus einem für elektromagnetische Wellen transparenten Kunststoffmaterial,
d a d u r c h g e k e n n z e i c h n e t ,
daß unmittelbar auf der Oberfläche des Elementes zumindest teilweise eine Mikrostrukturierung flächenhaft ausgebildet ist, bei der die Breiten und Abstände der einzelnen Strukturelemente kleiner als die kleinste Wellenlänge der mit dem Element zu beeinflussenden elektromagnetischen Wellen sind.

2. Optisch aktives Element nach Anspruch 1,
dadurch gekennzeichnet, daß freie Volumen innerhalb der Mikrostrukturierung mit wachsender Tiefe kontinuierlich abnimmt.

3. Optisch aktives Element nach Anspruch 1 oder 2,
dadurch gekennzeichnet, daß Strukturelemente der Mikrostrukturierung periodisch auf der Oberfläche ausgebildet sind und die Länge der Periode kleiner als die kleinste Wellenlänge der mit dem Element zu beeinflussenden elektromagnetischen Wellen ist.

4. Optisch aktives Element nach einem der Ansprüche 1 bis 3,
dadurch gekennzeichnet, daß die Mikrostrukturierung auf Oberflächen des Elementes ausgebildet ist/sind, in die die elektromagnetischen Wellen eingestrahlt werden und/oder austreten.

5. Optisch aktives Element nach einem der Ansprüche 1 bis 3,
dadurch gekennzeichnet, daß das Verhältnis von Tiefe und Periode der Mikrostruktur (Aspektverhältnis) nicht größer als 2:1 ist.

6. Optisch aktives Element nach Anspruch 4,
dadurch gekennzeichnet, daß das Verhältnis von Tiefe und mittlerer Breite der Strukturelemente der Mikrostruktur nicht größer als 2:1 ist.

7. Optisch aktives Element nach einem der Ansprüche 1 bis 6,
dadurch gekennzeichnet, daß das optische Element ein Prisma, eine konkav- oder konvexgeformte Linse, eine Zylinderlinse, ein Lentikular, eine Fresnellinse, ein Fresnelprisma, eine Fresnel-Zylinderlinse, ein Fresnelspiegel, der als Rückseitenspiegel verwendet wird, oder ein Strahlleiter ist.

8. Verfahren zur Herstellung eines optischen Elementes nach einem der Ansprüche 1 bis 7,
d a d u r c h g e k e n n z e i c h n e t ,
daß das optisch aktive Element mittels Spritzgießen in einem Werkzeug hergestellt wird, an dessen Oberfläche ein Negativabbild der Mikrostrukturierung ausgebildet ist.

9. Verfahren zur Herstellung eines optisch aktiven Elementes nach einem der Ansprüche 1 bis 7,
dadurch gekennzeichnet, daß das optisch aktive Element durch Heißprägen des bis über die Erweichungstemperatur erwärmten Kunststoffes mittels eines das negative Abbild der Mikrostrukturie-

rung aufweisenden Prägestempels hergestellt wird.

10. Verfahren zur Herstellung eines optisch aktiven Elementes nach einem der Ansprüche 1 bis 7, dadurch gekennzeichnet, daß die Mikrostrukturierung mit einem Laser durch Ablation auf der Oberfläche des Elementes ausgebildet wird.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DE 99/00945

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6: G 02 B 3/00, G 02 B 5/00, G 02 B 3/08, G 02 B 5/04, G 02 B 5/08, B 29 D 11/00, B 23 K 26/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6: B 23 K, B 29 D, G 02 B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4668558 A (BARBER, E.C.) 26 May 1987 (26.05.87) Column 2, line 27 – column 3, line 6, column 3, line 30 – column 4, line 42, column 23, line 17 – column 25, line 62, figure 9.	1-4, 7-9
X Y A	US 4013465 A (CLAPHAM et al.) 22 March 1977 (22.03.77) Column 1, line 7 – column 2, line 28, column 2, line 52 – column 3, line 43.	1-4 8, 9 5-7, 10
Y A	GB 2198279 A (NEC CORP.) 8 June 1988 (08.06.88) page 5, line 20 – page 8, line 7, page 9, line 19 – page 10, line 2, page 14, lines 3-25	8 1, 3
Y A	GB 1529021 A (SECRETARY OF STATE FOR INDUSTRY) 18 October 1978 (18.10.78), page 1, line 87 – page 2, line 110 (cited in the description).	9 1, 3, 4

[X] Further documents are listed in the continuation of box C. [] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 July 1999 (23.07.99)	3 September 1999 (03.09.99)
Name and mailing address of the ISA/E.P.O	Authorized officer Telephone No.

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/DE 99/00945

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES

G 02 B 3/00,G 02 B 5/00,G 02 B 3/08,G 02 B 5/04,
G 02 B 5/08,B 29 D 11/00,B 23 K 26/00

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK6

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)

B 23 K,B 29 D,G 02 B

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	US 4668558 A (BARBER, E.C.) 26. Mai 1987, Spalte 2, Zeile 27 - Spalte 3, Zeile 6, Spalte 3, Zeile 30 - Spalte 4, Zeile 42, Spalte 23, Zeile 17 - Spalte 25, Zeile 62, Fig. 9. --	1-4, 7-9
X	US 4013465 A (CLAPHAM et al.) 22. März 1977, Spalte 1, Zeile 7 - Spalte 2, Zeile 28, Spalte 2, Zeile 52 - Spalte 3, Zeile 43.	1-4
Y		8,9
A		5-7,10
	--	
Y	GB 2198279 A (NEC CORP.) 08. Juni 1988,	8

[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[] Siehe Anhang Patentfamilie

* Besondere Kategorien von angegebenen Veröffentlichungen :

"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist

"E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist

"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)

"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

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"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden

"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
23 Juli 1999	- 3. 09. 99

Name und Postanschrift der Internationale Recherchenbehörde	Bevollmächtigter Bediensteter
Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	GRONAU e.h.

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen PCT/DE 99/00945

III. EINSCHLAGIGE VERÖFFENTLICHUNGEN (Fortsetzung von Blatt 2)

Art *	Kennzeichnung der Veröffentlichung, soweit erforderlich unter Angabe der maßgeblichen Teile	Betr. Anspruch Nr.
	Seite 5, Zeile 20 - Seite 8, Zeile 7, Seite 9, Zeile 19 - Seite 10, Zeile 2, Seite 14, Zeilen 3-25.	
A		1,3
	--	
Y	GB 1529021 A (SECRETARY OF STATE FOR INDUSTRY) 18. Oktober 1978, Seite 1, Zeile 87 - Seite 2, Zeile 110 (in der Beschreibung genannt).	9
A		1,3,4

ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX

to the International Search Report to the International Patent Application No.

ANNEXE

au rapport de recherche international relatif à la demande de brevet international n°

PCT/DE 99/00945 SAE 234709

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

La présente annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visée ci-dessus. Les renseignements fournis sont donnés à titre indicatif et n'engagent pas la responsabilité de l'Office.

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